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# The Formation of Hydrogen Carriers by Haematin-Catalyzed Peroxidations

## I. HYDROGEN CARRIERS FROM CERTAIN ACRIDINE AND QUINOLINE COMPOUNDS

By A. ALBERT and J. E. FALK, *University of Sydney, Australia*

(*Received* 3 *June* 1948)

During studies of the inhibition of enzymes by antibacterial acridines, it was found that certain of these drugs caused a large stimulation of oxygen uptake in solutions containing ascorbic acid, cytochrome *c* and cytochrome oxidase. The same oxygen uptake occurred when the oxidase was omitted, and hence this enzyme played no part in the reaction. The studies which follow were, therefore, made in the absence of cytochrome oxidase.

Because the new effect interfered with the desired measurement of enzyme inhibitions, and, because it had possible importance in regard to the biological action of acridine compounds, its mechanism was studied in some detail. The effect occurred whenever certain amino- or hydroxy-acridines or analogous quinolines were shaken with ascorbic acid, cytochrome *c* and air. Most of the detailed studies which follow were made with 3-aminoacridine.

A brief outline of the apparent mechanism is given here. The new effect is characterized by a primary phase during which the rate of oxygen uptake increases to a maximum, and a secondary phase during which this maximum rate is maintained until the substrate is exhausted, whereupon the uptake of oxygen ceases. The initiation of the effect depends on a trace of hydrogen peroxide (the origin of which is discussed below)which, in the presence of cytochrome *c*, brings about a catalyzed peroxidative oxidation. For example, 3-aminoacridine is oxidized to an *o*-hydroxyaminoacridine which is, in turn, autoxidizable to an *o*-quinonimine with production of hydrogen peroxide. By utilization of this hydrogen peroxide in the peroxidative reaction the effect becomes autocatalytic. The *o*-quinonimine acts as a hydrogen carrier in the oxidation of the substrate and oxygen is the ultimate hydrogen acceptor.

A similar series of reactions was found to occur when cytochrome *c* was replaced by methaemoglobin, so that the initial reaction is evidently a nonspecific haematin peroxidative effect. Similar effects were found with cysteine as substrate but not with hydroquinone. There was no relation between the antibacterial activity of the acridine and quinoline compounds and their ability to give the effect.

## EXPERIMENTAL

*Materials.* Ascorbic acid was used as the pure substance, kindly supplied by the Colonial Sugar Refining Co., Sydney; it was determined by titration with 2:6-dichlorophenolindophenol (cf. Lemberg, Cortis-Jones & Norrie, 1938). Cysteine was the Schering-Kahlbaum copper-free preparation. Hydrogen peroxide (100 vol.) was freshly diluted before use and standardized with 0·01 N-$KMnO_4$. Water for all purposes was double distilled from an all glass still. The catalase was a preparation from horse liver, with a *Kat.-f.* of 25,000. The cytochrome *c* contained 0·33% Fe and was obtained from ox heart by method (*a*) of Keilin & Hartree (1945). It was dialyzed against distilled water in Tee-Pak synthetic sausage casings (Harvey Little and Co., Sydney) which adsorbed very little cytochrome. The catalase-free methaemoglobin solution was prepared as follows. A solution of pure crystalline haemin in dilute $Na_2CO_3$ was accurately determined spectrophotometrically as pyridine haemochromogen. A solution of native human globin was prepared by the method of Anson & Mirsky (1925) and its protein content determined. The solutions of haemin and globin were mixed so that there was 10% excess of globin. For the manometric experiments a volume of this solution equivalent to 1·0 mg. of methaemoglobin was used. In terms of Fe content, this was equivalent to 1 mg. of cytochrome *c*.

Aniline (b.p. 184°) and phenol (b.p. 182°) were freshly distilled. The m.p. of the *o*-phenylenediamine was 103°; *p*-phenylenediamine, 139°; catechol, 104°; *o*-aminophenol, 170° and *p*-aminophenol, 184°. The preparations and m.p.'s of the acridine compounds listed in Table 3 were as given by Albert, Rubbo, Goldacre, Davey & Stone (1945), and of the quinoline compounds as given by Albert & Magrath (1947) with the following additions: 3:5-dihydroxyacridine (m.p. 347–348°) was prepared according to Ullmann & Kipper (1907); 5-aminoquinoline (m.p. 110°) by the hydrogenation of 5-nitroquinoline over Raney nickel; 5-hydroxyquinoline (m.p. 223°) by the method of Claus & Howitz (1893) and 5-amino-6-hydroxyquinoline (m.p. 195°, decomp.) by the method of Mathëus (1888). Phenol blue, *p*-dimethylaminoaniline hydrochloride and *p*-diethylaminoaniline hydrochloride were obtained from British Drug Houses Ltd.

*Methods.* The measurements of $O_2$ uptake were made in Warburg respirometers at pH 7·0 and 25 or 37·5°. The shaking rate was 120/min., the gas phase usually air, and, in the total liquid volume of 2·0 ml., the phosphate buffer was 0·12 M; changing the buffer concentration did not affect the results. Experimental pH values were checked with a glass

electrode. All solutions were prepared just prior to use. The basic compounds were dissolved by the aid of 1–1·5 equiv. of HCl, and the hydroxy compounds, when necessary, with 1 equiv. of NaOH. The ascorbic acid was dissolved in water, with the addition of 1 equiv. of 0·1 N-NaOH, just before pipetting.

## RESULTS

### *The effect*

The nature of the effect is summarized in Fig. 1, curve 1 showing the typical autocatalytic reaction. When the ascorbic-acid concentration was halved, the total oxygen uptake was also approximately halved (curve 2).

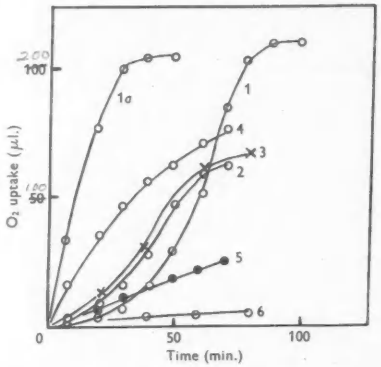



Fig. 1. The effect obtained with 3-aminoacridine, sodium ascorbate and cytochrome *c* at pH 7·0 and 25°. Curve 1: 3-aminoacridine, ascorbate and cytochrome *c* (concentrations in Table 1). Curve 1*a*: uptake on addition of a second lot of ascorbate after reaction 1. Curve 2: as curve 1 with ascorbate concentration halved. Curve 3: the rate of utilization of ascorbate determined by titration (see text). Curve 4: 80% of the precipitate from reaction 1, isolated as described in text, and allowed to react with ascorbate only; addition of cytochrome *c* made no difference. Curve 5: autoxidation controls, with ascorbate. Curve 6: autoxidation controls without ascorbate.

When the reaction was stopped at successive time intervals by the addition of trichloroacetic acid, and the amount of ascorbic acid used determined by titration, the rate of its utilization was parallel to the rate of oxygen uptake; in curve 3 this rate is plotted as the calculated amount of oxygen required to oxidize (to dehydroascorbic acid) the ascorbic acid used up in the given times. The calculated amount of oxygen required to oxidize all the ascorbic acid to dehydroascorbic acid was 125 μl., and the total uptake in curve 1 shows that it was not oxidized past this stage. In a small number of experiments with cysteine as substrate, a similar effect was found, the total oxygen uptake then being only slightly more than that required to produce cystine. Hydroquinone gave no oxygen uptake at all, although it is a substrate for the full cytochrome *c*- cytochrome oxidase system.

When the effect occurred, a dark-coloured precipitate was usually observed at the end of the reaction. This precipitate dissolved, with loss of the dark colour, on addition of further ascorbic acid, and reappeared when this had been oxidized. It was similarly dissolved by the addition of cysteine or sodium dithionite (hydrosulphite). The precipitate, isolated by centrifuging and washed well at the centrifuge with cold phosphate buffer, pH 7·0, catalyzed the oxidation of ascorbic acid by oxygen (curve 4). Addition of cytochrome *c* made no difference to the rate, unless some unchanged test substance was added with an incompletely washed precipitate, in which case cytochrome *c* caused a significant increase. The precipitate lost its catalytic activity entirely when kept in the buffer for 2 days at 0°. Its colour, its behaviour on reduction and re-oxidation and its instability strongly suggested a substance of quinonoid type.

Table 1. *The effect of varying the concentration of 3-aminoacridine and of cytochrome* c, *at* pH 7·0 *and* 25°

(Each flask contained hydrogen peroxide, $10^{-3}$ M, sodium diethyldithiocarbamate $6{\cdot}4 \times 10^{-3}$ M, and sodium ascorbate $5{\cdot}6 \times 10^{-3}$ M, as well as, in experiment (*a*), cytochrome *c* $2 \times 10^{-5}$ M and, in experiment (*b*), 3-aminoacridine $10^{-3}$ M.)

(*a*)

| 3-Aminoacridine M | $O_2$ uptake in 30 min. (μl.) |
|---|---|
| 0 | 0 |
| $10^{-5}$ | 12 |
| $10^{-4}$ | 52 |
| $10^{-3}$ | 98 |

(*b*)

| Cytochrome *c* M × $10^{-5}$ | |
|---|---|
| 0 | 0 |
| 0·18 | 14 |
| 0·54 | 47 |
| 0·90 | 79 |
| 1·80 | 105 |

It was important to determine whether the precipitate contained cytochrome *c* or a derivative, e.g. an autoxidizable haem compound. No absorption band whatever could be seen in a suspension of well washed, active precipitate, or in a solution made by reducing it with sodium dithionite. Nor could iron be detected in the precipitate after digestion with concentrated sulphuric acid and hydrogen peroxide. If the precipitate were a compound or mixture of 3-aminoacridine and cytochrome *c* in molecular ratios 100:1, iron could readily have been detected under the conditions of the test. The precipitate must, therefore, be an iron-free derivative of 3-aminoacridine.

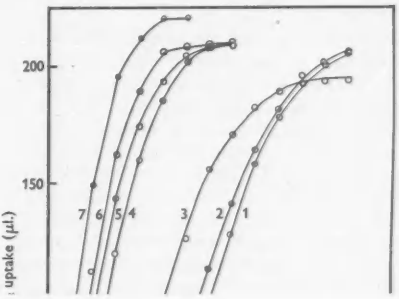
*Studies of the mechanism*

In the presence of either cytochrome *c* or methaemoglobin, the effect was inhibited by catalase on the one hand (Table 2), and stimulated by hydrogen peroxide on the other (Fig. 2). In the absence of a haematin compound the effect did not occur, and was not induced by hydrogen peroxide (Table 2). There was thus strong evidence for the occurrence of a haematin-catalyzed peroxidative reaction.

Under certain conditions, either the 3-aminoacridine concentration or the cytochrome *c* concentration could limit the rate of reaction (Table 1). In these experiments hydrogen peroxide was added so that there was no lag, and the initial rates were comparable.

Cytochrome *c* does not combine with carbon

of this hydrogen peroxide. If this was a limiting factor, the rate during the autocatalytic stage would be depressed and the lag lengthened. This was




## CORRIGENDA

**The formation of hydrogen carriers by haematin-catalysed peroxidations. 1**

**By A. Albert and J. E. Falk**

**Volume 44 (1949), No. 2**

**p. 130, Fig. 1, *y* axis '$O_2$ uptake (μl.)':**
***for* scale marks 50 and 100 *read* 100 and 200**

**p. 130, col. 1, line 4 from end:**
***for* it was not oxidized *read* it was oxidized**

well as by diethyldithiocarbamate. The latter reagent also slightly depressed the effect in the presence of cytochrome *c* (Table 2). The autoxidation of ascorbic acid produces hydrogen peroxide, and the CO and diethyldithiocarbamate inhibitions of the effect may be due to their prevention of the formation

Fig. 2. The effect obtained with 3-aminoacridine and different concentrations of hydrogen peroxide at pH 7·0 and 25°. Each flask contained 3-aminoacridine, cytochrome *c* and ascorbate with concentrations as in Table 1. Curves 1–7: hydrogen peroxide: nil, 0·01, 0·1, 0·25, 0·5, 1·0 and 10·0 × $10^{-4}$ M respectively.

Table 2. *The effect of various reagents on the rate of oxygen uptake of ascorbic acid with 3-aminoacridine at* pH 7 *and* 37·5°

(Each flask contained, in separate side arms, sodium ascorbate (final molarity 5·6 × $10^{-3}$) and 3-ami noacridine (fina molarity 2 × $10^{-3}$). These were tipped on to the reagents indicated in column 1 after equilibration. Molarity of cytochrome *c*, 0·9 × $10^{-5}$; of methaemoglobin, 0·22 × $10^{-5}$; of $H_2O_2$, 1·0 × $10^{-3}$; of $CuSO_4$, 2·0 × $10^{-5}$; of sodium diethyldithiocarbamate 6·4 × $10^{-3}$. Values of $t$ (time to reach maximum rate) were accurately reproduced to ±3 min., and of $v$ (the maximum rate) to at least ±0·5 μl./min., accuracy increasing as $v$ became smaller.)

| Added to main bulb | Gas phase | $t$ (min.) | $v$ (μl. $O_2$/min.) |
|---|---|---|---|
| Nil | Air | 0 | 0·5 |
| Nil | CO-$O_2$ | 0 | 0 |
| Diethyldithiocarbamate | Air | 0 | 0 |
| Cytochrome *c* | Air | 35 | 5·5 |
| Cytochrome *c* | CO-$O_2$ | 55 | 3·0 |
| Cytochrome *c* + $H_2O_2$ | Air | 0 | 18·3 |
| Cytochrome *c* + catalase | Air | 65 | 2·0 |
| Cytochrome *c* + diethyldithiocarbamate | Air | 43 | 5·0 |
| Cytochrome *c* + $CuSO_4$ | Air | 0 | 5·0 |
| Cytochrome *c* + $CuSO_4$ | CO-$O_2$ | 0 | 4·0 |
| Methaemoglobin | Air | 45 | 3·6 |
| Methaemoglobin | CO-$O_2$ | 60 | 2·9 |
| Methaemoglobin + $H_2O_2$ | Air | 0 | 18·0 |
| Methaemoglobin + catalase | Air | 0 | 0·6 |

indirectly confirmed by addition of small amounts of copper sulphate. As shown in Table 2, the lag was abolished; in the presence of copper the reaction was again inhibited to some extent by carbon monoxide.

Although hydrogen peroxide could arise from autoxidation of the ascorbic acid, it was realized that this was not necessarily the source of the peroxide which initiated the effect. The effect still occurred in the presence of carbon monoxide or diethyldithiocarbamate, although autoxidation was then negligible. In addition, when the 3-aminoacridine was shaken with the cytochrome *c* for 30 min. before adding the ascorbic acid, considerable darkening in colour had occurred, and the lag was shorter than when the reagents were mixed immediately after equilibration as was usual. This darkening was inhibited by catalase.

This formation of carrier in the absence of ascorbic acid suggested that a trace of hydrogen peroxide was introduced from another source. It was found that the cytochrome *c* solutions, prepared as above, gave an output of oxygen gas on the addition of catalase. It is not yet known at what stage of the preparation this peroxide arose, and, in view of the importance of the presence of this unsuspected contaminant in preparations of cytochrome *c* made in accordance with published instructions, this question is now under investigation.

*The chemical change in the peroxidative reaction*

The following (I, II, III) are the reversible hydrogen-carrying systems which appear most likely to be formed by oxidation of 3-aminoacridine.

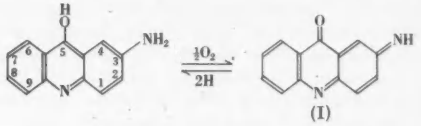
In order to explore these possibilities, it was decided to examine the series of acridine and quinoline compounds given in Table 3. The ionization (as bases) under the conditions of the experiment have been included in the table as this was found to be relevant. The antibacterial activities of these compounds are also recorded to show that the well-known antibacterial properties of some members of these series do not run parallel to their ability to become carriers.

The results obtained from the acridine compounds are given in Table 4 and those from the quinoline compounds in Table 5.

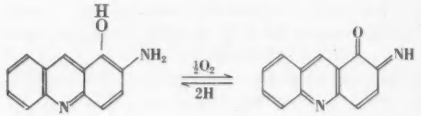
Compounds of the types (I–III) could be formed from any of the acridine compounds which gave the effect (Table 4); quinones would be the corresponding oxidized forms of the carriers derived from hydroxyacridines.

Carriers of type (I) were excluded, however, since neither 3-amino-5-hydroxyacridine nor 3:5-dihydroxyacridine (nos. 15 and 16) had any carrier activity. Oxidation in the position ortho to the existing amino or hydroxyl group thus seemed more likely.

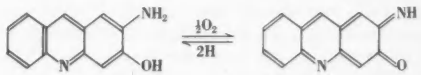
Since acridine compounds of types (II) and (III) are unknown and likely to be extremely difficult of access, attention was concentrated on the quinoline




(I)

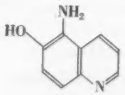



(II)




(III)

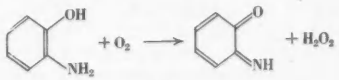
compounds (Table 5). From 5-aminoquinoline, which gave an effect similar to 3-aminoacridine, only one ortho-substituted compound (IV) is possible.




(IV)

This compound, 5-amino-6-hydroxyquinoline, was synthesized, and was found to act directly as a reversible hydrogen carrier in the oxidation of ascorbic acid; 7-amino-8-hydroxyquinoline acted similarly, as also did the indamine dye, phenol blue.

There is thus good reason to believe that the carrier formed from 5-aminoquinoline is the readily autoxidizable substance, 5-amino-6-hydroxyquinoline. It became important to determine whether, as assumed above, autoxidation of compounds of this type gives rise to hydrogen peroxide (as in V).




(V)

When solutions in phosphate buffer, pH 7, of 5-amino-6-hydroxyquinoline and 7-amino-8-hydroxyquinoline were shaken with air at 25 or 37·5°, there was with the latter compound a fast uptake of oxygen, the full theoretical amount being consumed in about 1 hr. The former consumed oxygen more slowly, and, in 1 hr., only 75% of the theoretical amount had been taken up. When catalase was added after 1 hr., there was in each case an oxygen output

Table 3. *The acridine and quinoline compounds used, their strength as bases and their antibacterial activity (where known)*

| No. | Compound | $pK_a$, basic, 25° in water | Percentage ionized as base, 25° pH 7 (*g*) | B.I. (*h*) |
|---|---|---|---|---|
| 1 | Acridine | 4·52 (*a*) | 0·34 | 6 |
| 2 | 1-Aminoacridine | 4·33 (*a*) | 0·22 | 4 |
| 3 | 2-Aminoacridine | 7·94 (*a*) | 89·70 | 21 |
| 4 | 3-Aminoacridine | 5·80 (*a*) | 5·94 | 8 |
| 5 | 4-Aminoacridine | 5·96 (*a*) | 8·37 | 9 |
| 6 | 5-Aminoacridine | 9·88 (*a*) | 99·86 | 25 |
| 7 | 2:7-Diaminoacridine | 8·02 (*b*) | 91·2 | 26 |
| 8 | 2:8-Diaminoacridine | 9·54 (*b*) | 99·4 | 22 |
| 9 | 2:8-Diamino-1:9-dimethylacridine | 9·05 (*c*) | 99·11 | 30 |
| 10 | 2:8-Diamino-3:7-dimethylacridine | 10·23 (*c*) | 99·94 | 21 |
| 11 | 2:8-Bisdimethylaminoacridine | 10·57 (*c*) | 99·97 | 17 |
| 12 | 3-Amino-5-methylacridine | 6·14 (*c*) | 12·23 | 10 |
| 13 | 3-Hydroxyacridine | 4·63 (*d*) | 0·43 | 0 |
| 14 | 5-Hydroxyacridine (acridone) | — | <0·01 (*d*) | 0 |
| 15 | 3:5-Dihydroxyacridine | — | <0·01 | — |
| 16 | 3-Amino-5-hydroxyacridine | — | — | — |
| 17 | 5-Aminoquinoline | 5·43 (*e*) | 2·35 | 0 |
| 18 | 6-Aminoquinoline | 5·54 (*e*) | 3·36 | 0 |
| 19 | 5-Hydroxyquinoline | — | — | 0 |
| 20 | 8-Hydroxyquinoline | 4·95 (*f*) | 0·95 | 26 |
| 21 | 5-Amino-6-hydroxyquinoline | — | — | — |
| 22 | 7-Amino-8-hydroxyquinoline | — | — | — |

(*a*) Calculated from values in water at 20° (Albert & Goldacre, 1946), by a temperature correction found by interpolation in a curve based on the data of Hall & Sprinkle (1932).

(*b*) Kindly determined for this work by Mr J. N. Phillips.

(*c*) Calculated from values in 50% ethanol (Albert & Goldacre, 1946), by adding 0·64, the mean depression by ethanol for 11 compounds (Albert & Goldacre, 1946). Temperature correction as in (*a*) then applied.

(*d*) Calculated from data of Albert & Goldacre (1943).

(*e*) Calculated from data of Albert & Goldacre (1944).

(*f*) Calculated from data of Albert & Magrath (1947).

(*g*) Calculated from the equation: $\%\ \text{ionized} = \frac{100}{1+\text{antilog}\ (\text{pH}-\text{pK}_a)}$.

(*h*) The Bacteriostatic Index (from Albert *et al.* 1945; Albert, Rubbo, Goldacre & Balfour, 1947). A B.I. of 15 indicates significant antibacterial activity, and a B.I. of 20 or higher, marked activity.

Table 4. *The results of testing various acridine compounds*

(The compounds were tested with ascorbic acid and cytochrome *c*, with air as gas phase, with concentrations and conditions as in Table 1. Where the effect was found, the reactions when hydrogen peroxide or catalase was added were similar to those shown in Table 2. Hydrogen peroxide did not induce the effect in the compounds in column 2.)

| No. | (1) Compounds which gave the effect at pH 7 | No. | (2) Compounds which were completely inactive |
|---|---|---|---|
| 4 | 3-Aminoacridine | 1 | Acridine |
| 5 | 4-Aminoacridine | 2 | 1-Aminoacridine |
| 7 | 2:7-Diaminoacridine | 6 | 5-Aminoacridine |
| 8 | 2:8-Diaminoacridine | 10 | 2:8-Diamino-3:7-dimethylacridine |
| 9 | 2:8-Diamino-1:9-dimethylacridine | 11 | 2:8-Bisdimethylaminoacridine |
| 13 | 3-Hydroxyacridine | 12 | 3-Amino-5-methylacridine |
| 3 | 2-Aminoacridine (at pH 8·5) | 14 | 5-Hydroxyacridine (acridone) |
| | | 15 | 3:5-Dihydroxyacridine |
| | | 16 | 3-Amino-5-hydroxyacridine |

Table 5. *The results of testing various quinoline compounds*

(The compounds in column 2 were directly active as carrers in the oxidation of ascorbic acid; the *v* values were for the $O_2$ uptake of $5{\cdot}6 \times 10^{-3}$ M ascorbic acid with the carrier $2 \times 10^{-3}$ M, at pH 7·0 and 25°; there was no lag, and addition of cytochrome *c* made no difference. Of these compounds, only nos. 18 and 22 gave a precipitate (see text).)

| No. | (1) Compounds which gave the effect | No. | (2) Compounds which were active even in the absence of cytochrome *c* | *v* (μl. $O_2$/min.) |
|---|---|---|---|---|
| 17 | 5-Aminoquinoline | 21 | 5-Amino-6-hydroxyquinoline | 17·2 |
| 18 | 6-Aminoquinoline | 22 | 7-Amino-8-hydroxyquinoline | 18·0 |
| 19 | 5-Hydroxyquinoline | | Phenol blue (for comparison) | |
| 20 | 8-Hydroxyquinoline | | | |

corresponding to half the oxygen consumed as required by (V) and the equation,

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}O_2.$$

That hydrogen peroxide is formed on autoxidation of these compounds at pH 7 was thus established. When *o*- and *p*-aminophenol were tested in the same way, however, no oxygen uptake or output was found with either.

The failure of some of the acridine compounds to give the effect must now be considered. The failure of 2-aminoacridine to yield any carrier in the usual test at pH 7 was surprising, in view of the activity of its derivatives, nos. 7 and 8 (Table 4). 3-Aminoacridine at pH 7 is mainly un-ionized, while 2-aminoacridine is ionized to the extent of 89·7 %. Although 2:8-diaminoacridine exists almost entirely in the cationic form at pH 7, it is likely that a significant proportion of one amino group remains un-ionized. In 2:7-diaminoacridine, the 7-amino group does not enter into the ionic resonance and is entirely un-ionized (Albert & Goldacre, 1946). It thus seemed possible that 2-aminoacridine might give the effect if it could be tested with a higher proportion of its amino group in the un-ionized form. This proved to be the case (Table 6). At pH 8·5, when it was 78 % un-ionized, it gave a carrier effect similar to that of 3-aminoacridine.

Although, in this group of substances, the presence of an un-ionized amino group appears to be one of the prerequisites for carrier formation, other factors also operate. For example, at pH 7 no. 2 is almost completely un-ionized but gives no carrier (Table 4).

A few benzenoid compounds were tested to find whether the effect would also occur in this series. The result was negative. Aniline, phenol, catechol, hydroquinone and *o*-phenylenediamine gave no carriers under any conditions. Phenol blue and 2:6-dichlorophenolindophenol acted directly as carriers in the well known manner, no cytochrome *c* being required. *p*-Phenylenediamine, *p*-dimethylaminoaniline, *p*-diethylaminoaniline and *p*-aminophenol all gave carriers which acted similarly to phenol blue in Table 5, provided that they were shaken with cytochrome *c* at 25° and pH 7 for 0·5 hr. before adding the ascorbic acid. Under these conditions they were oxidized to coloured compounds; on adding the ascorbic acid the colour disappeared, but returned when the ascorbic acid had been oxidized. In the case of the first three compounds it was probable that the carriers were the Wurster's salts which they are known to yield on oxidation. It seemed that ascorbic acid, if added too soon, protected them from oxidation to this form.

## DISCUSSION

For the effect to occur, it is evidently necessary that: (*a*) A peroxidative oxidation should take place in such a way that a hydroxyl group enters the acridine or quinoline nucleus ortho (or, conceivably, para) to an existing amino or hydroxyl group. (*b*) The product of this reaction should be of suitable oxidation-reduction potential to act as a reversible hydrogen carrier in the oxidation of ascorbic acid by oxygen.

In Table 4, a number of compounds which did not give the effect are shown. Comparison of nos. 4 and 12 is interesting; the 5-methyl group in no. 12 should make little difference to its oxidizability ortho to the amino group (i.e. in the 2- or 4-positions), or to the oxidation-reduction potential of the oxidized compound, but it is possible that the methyl group sterically hindered the peroxidative reaction. Similar considerations apply to no. 10 (compare no. 9) and to nos. 15 and 16 which have been discussed above. No. 6 has no oxidizable position ortho to its amino group.

The 3-aminoacridine effect appears to depend on the mechanism shown in the scheme on p. 135.

In the case of the hydroxy compounds (e.g. 3-hydroxyacridine) the reduced form of the reversible system is probably an *o*-dihydric phenol, and the oxidized form an *o*-quinone.

Evidence has been found that solutions of cytochrome *c*, prepared by the method of Keilin & Hartree (1945), may contain small amounts of hydrogen peroxide. This could make a considerable difference to *in vitro* studies, for example by initiating coupled oxidations, such as have been described above. Work which is proceeding on this subject will be reported separately.

Some examples will now be mentioned in which previous workers seem to have met the effect reported here without having recognized its true nature.

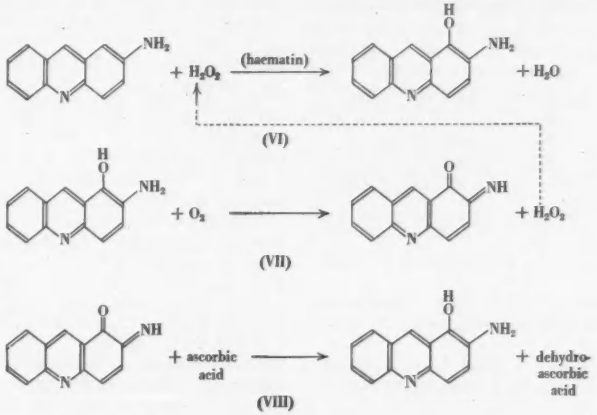
Table 6. *The effect with 2-aminoacridine at* pH 8·5 *and* 25°

(Each flask contained test substance, ascorbate and cytochrome *c* in the concentrations shown in Table 2. Borate buffer, pH 8·5 (0·035 M) was used.)

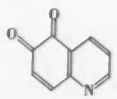
| | *t* (min.) | *v* (μl. $O_2$/min.) | Percentage un-ionized at pH 8·5 | Percentage un-ionized at pH 7·0 |
|---|---|---|---|---|
| 2-Aminoacridine | 120 | 0·8 | 78·0 | 10·3 |
| 2-Aminoacridine with $H_2O_2$, $10^{-3}$ M | 0 | 5·0 | — | — |
| 3-Aminoacridine | 60 | 1·6 | 99·8 | 94·1 |
| 3-Aminoacridine with $H_2O_2$, $10^{-3}$ M | 0 | 10·5 | — | — |

Ames, Ziegenhagen & Elvehjem (1946), using 8-hydroxyquinoline as a copper-removing reagent in systems containing cytochrome oxidase, cytochrome *c* and ascorbic acid, reported a 'non-enzymic reaction in which a rapid oxygen uptake masked the enzyme reaction'. This was without doubt due to the reaction of 8-hydroxyquinoline as described above (cf. Table 5). A similar stimulation of oxygen uptake was found by Stotz, Harrer, Schultze & King (1937) when 8-hydroxyquinoline was added to washed liver brei to which ascorbate and haemolyzed blood had been added. Washing the tissue removed blood pigments and other haematin compounds, and in the absence of haemolyzed blood no stimulation was found. From the work of Lemberg, Legge & Lockwood (1939), the oxygen uptake on incubation of haemolyzed blood with ascorbate at pH 7 is known to be due to a coupled oxidation resulting in choleglobin formation; this process is stimulated by hydrogen peroxide and inhibited by catalase. Stotz *et al.* (1937) observed green pigment formation when 8-hydroxyquinoline was present, and it has since been found (Mr E. C. Foulkes, private communication) that choleglobin formation is markedly accelerated on addition of the carrier 7-amino-8-hydroxyquinoline (Table 5) to haemolyzed blood incubated with ascorbate at pH 7 and 37°. The influence of 8-hydroxyquinoline may be explained by assuming preliminary peroxidative oxidation of the 8-hydroxyquinoline to a hydrogen carrier, which catalyzes the oxidation of the ascorbate with increased rate of oxygen uptake as described by Stotz *et al.* The peroxide produced in this carrier-catalyzed oxidation should accelerate choleglobin formation, and the same explanation would apply to the experiment with pre-formed carrier.

Tauber (1936) showed that horseradish peroxidase catalyzes the oxidation of ascorbic acid by hydrogen peroxide only if 'substances capable of forming quinones are present', his examples of the latter being vanillin, *p*-phenylenediamine and fresh adrenal extract (but not adrenaline).




The possibility must be kept in mind that carriers of these types may also be formed *in vivo*, the introduction of hydroxyl groups into benzenoid rings being a well-known biological mechanism. Specifically, Fühner (1904) found that acridine fed to rabbits was excreted in the urine as 3:5-dihydroxyacridine and later Fühner (1906) showed that when quinoline was given to man a substance appeared in the urine which oxidized in air to the *o*-quinone (IX):



(IX)

Schönhöfer (1942) found an interesting relationship among aminoquinoline compounds, in that gametocidal antimalarial activity was linked with the possibility of their biological conversion to quinonoid compounds.

## SUMMARY

1. Cytochrome *c* and methaemoglobin catalyze (*in vitro*) the oxidation, by hydrogen peroxide, of certain amino- and hydroxy-acridines and -quinolines.

2. The oxidized products are hydrogen carriers which efficiently promote the autoxidation of ascorbic acid or cysteine.

3. The oxidized forms of the carriers appear, in the case of the amino compounds, to be *o*-quinonimines and, in the case of hydroxy compounds, *o*-quinones.

4. The mechanism of the formation and action of the carriers is discussed.

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# Metabolism of Derivatives of Toluene

## 2. NUCLEAR METHYL-SUBSTITUTED DERIVATIVES OF *N*-PHENYLUREA

BY H. G. BRAY, H. J. LAKE AND W. V. THORPE
*Department of Physiology, Medical School, University of Birmingham*

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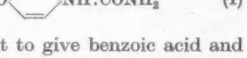
The compounds included in this study were *N*-phenylurea and *N*- (*o*-, *m*- and *p*-tolyl)ureas. The metabolism of these substances does not appear to have been investigated previously, although two drugs which may be regarded as derivatives of *N*-phenylurea have received attention. 'Elbon' (I, $R = C_6H_5$) was shown by Morinaka (1922) to be



(I)

hydrolyzed in the rabbit to give benzoic acid and *N*-(*p*-hydroxyphenyl)urea, the former being excreted as hippuric acid and the latter as ether glucuronide, which was obtained as a crystalline potassium salt. Tsunoo (1935) showed that 'Ethynal', the corresponding 2-furyl derivative (I, $R = C_4H_3O$) gave rise in the dog to 2-(2′-furyl)-acrylic acid (excreted as such, and as its glycine conjugate) and the glucuronide of *N*-(*p*-hydroxyphenyl)urea; in the rabbit its fate was believed to be similar, although only the glucuronide was isolated. In man the only metabolite identified was furan-2-carboxylic acid (α-furoic acid).

There appears to be no account in the literature of the behaviour *in vivo* of the ureido group (—NH.CO.$NH_2$) attached to an aromatic ring, except for the two papers mentioned above, although compounds containing it were often claimed as metabolites of amino compounds by earlier workers. Such 'uramino' compounds include those stated to be derived from sulphanilic acid (Ville, 1892), anthranilic acid (Mitsuba & Ichihara, 1927), *m*-aminobenzoic acid (Salkowski, 1882) and various aminohydroxybenzoic acids (Pruszynski, 1893). All these compounds were believed to have been formed by the conjugation of urea with the amino group of the substance administered, ammonia being eliminated. These observations have, however, not been repeated in more recent investigations and it now appears likely that the compounds were artefacts, formed during isolation, which, in most early investigations, involved evaporation of the urine to dryness (cf. Schmidt & Allen (1920) on Salkowski's (1873) uramino compound from taurine; Friedmann (1931) on excretion products of α-naphthylalanine and Bray, Lake, Neale, Thorpe & Wood

(1948*b*) on aminobenzoic acids). This process is now rarely used, having been superseded by direct extraction of the urine with ether or other solvents in continuous extractors.

It has been shown that neither acetylation nor alkylation of the amino group of aniline alters the position in which the hydroxyl group enters as a result of biological hydroxylation: aniline and acetanilide both give the para hydroxy derivative and acetanilide also gives the ortho compound (Schmiedeberg, 1878; Müller, 1887; Jaffé & Hilbert, 1888; Mörner, 1889); *NN*-dimethyl- and *NN*-diethyl-anilines both give para hydroxy derivatives in the rabbit (Horn, 1936, 1937). It was, therefore, of interest to determine whether modification of the amino group as it occurs in *N*-phenylurea derivatives has any influence different from acetylation and alkylation.

The metabolites which might be expected from phenylurea and the tolylureas would be the free amino compounds or their acetyl derivatives, hydroxylated derivatives either free or conjugated with glucuronic or sulphuric acid, and, in the case of the tolylureas, the corresponding benzoic acids formed owing to the oxidation of the methyl group. These acids might be free or conjugated with glucuronic acid or glycine.

The investigation carried out was similar to that described in Part I of this series (Bray & Thorpe, 1948), including a quantitative study of the effects of the four compounds upon the excretion of ether-soluble acid, diazotizable material, glucuronides and ethereal sulphates by the rabbit. Qualitative studies included the isolation of oxidation and hydroxylation products similar in nature to those obtained from the acetotoluidides.

## MATERIALS AND METHODS

*Materials.* The *N*-phenylurea derivatives necessary for this investigation were readily prepared by the interaction of aqueous solutions of NaCNO and of the hydrochloride of the corresponding amino compound. It was found that at least three recrystallizations from aqueous ethanol were necessary to give a product which gave a negligible colour on diazotizing and coupling. Commercial preparations (*N*- (*o*- and *m*-tolyl)urea (Genatosan) and *N*-phenylurea (Light and Co.)) required similar treatment. The two *N*-(hydroxymethylphenyl)ureas were prepared from the corresponding aminocresols (Bray & Thorpe, 1948) and do not appear to have been described previously. N-(4-*hydroxy*-2-*methylphenyl*)*urea* formed large colourless prisms, m.p. 232°, which gave a transient weak blue colour with $FeCl_3$; the diazo reaction was negative, although a faint blue colour developed after 30 min. The compound gave no indophenol reaction. (Found: N, 16·9. $C_8H_{10}N_2O_2$ requires N, 16·89%.) N-(4-*hydroxy*-3-*methylphenyl*)*urea* formed white needles, m.p. 203°. It gave a transient faint brown coloration with $FeCl_3$; diazo and indophenol reactions were negative. (Found: N, 16·9. $C_8H_{10}N_2O_2$ requires N, 16·89%.)

It was found that most phenylurea derivatives on keeping, either as solid or in solution, were hydrolyzed so that diazotizable material could be detected. Table 1 shows results obtained with *N*-phenylurea. The extent of this hydrolysis was somewhat greater in the case of *m*- and *p*-ureidobenzoic acids and somewhat less with the three tolylureas. It was for this reason that the compounds administered to the rabbits were recrystallized from aqueous ethanol immediately before an experiment. In some cases several recrystallizations failed to give a product entirely free from diazotizable material. Control experiments in which phenylurea derivatives were left standing in normal rabbit urine for 20 hr. gave results for free amino compound (by diazo reaction) corresponding to about 2%. It is, therefore, concluded that the free amino values observed in the experimental urines (Tables 2–5) are probably due to spontaneous hydrolysis rather than to any metabolic changes.

Table 1. *Spontaneous decomposition of N-phenylurea at 20°*

| Time (hr.) | Percentage conversion to aniline: Aqueous solution (219 mg./100 ml.) | Percentage conversion to aniline: Solid recrystallized from aqueous ethanol |
|---|---|---|
| 0 | — | 0 |
| 0·5 | 0·5 | — |
| 2 | 0·5 | 0·4 |
| 19 | 1·9 | 0·8 |
| 98 | 3·2 | 3·1 |
| 196 | 7·2 | 7·7 |

The two *N*-(hydroxymethylphenyl)ureas are more stable in the solid state. When heated in solution some decomposition occurs, especially with *N*-(4-hydroxy-3-methylphenyl)-urea, which could only with difficulty be obtained with the ureido group intact by mild hydrolysis of its glucuronide (see p. 140).

*Diet and dosage.* The rabbits used were does of 2–3 kg. The diet of rabbit pellets (Bruce & Parkes, 1946) and water previously described (Bray, Ryman & Thorpe, 1947) was maintained throughout the study. The compounds investigated were administered by stomach tube as suspensions in water. None of the tolylureas showed any toxic effect at a dose level of 0·25–0·30 g./kg., but phenylurea was appreciably toxic at this level, the rabbits recovering completely in a few hours.

*Methods of analysis.* These were essentially the same as those used in the acetotoluidide study (Bray & Thorpe, 1948), but included also estimation of the reducing value of urine hydrolyzed for 1·5 hr. with HCl (cf. Bray, Neale & Thorpe, 1946). The hydroxylation products formed as metabolites of phenylurea and *o*-tolylurea did not reduce the Shaffer-Hartmann reagent to any appreciable extent either before or after hydrolysis under the conditions used. The hydroxylation product of *m*-tolylurea was, however, slightly reducing after hydrolysis (3·44 mg. ≡ 1 mg. glucose) and allowance for this was made in the same way as for hydroxysulphapyridine (Bray *et al.* 1946). Estimation of diazotizable material in hydrolyzed urine was not carried out owing to the difficulty of finding conditions which would give quantitative results. The ureido compounds are not completely hydrolyzed under Bratton & Marshall's (1939) conditions: even a tenfold increase in concentration

of acid failed to achieve complete hydrolysis. (This is surprising in view of the spontaneous hydrolysis of these compounds already referred to.) The use of this more concentrated acid is attended by the difficulty of adjusting the acidity to that suitable for development of the diazo colour which is retarded or inhibited by excess of acid. Another source of error is that, unless suitable precautions are taken, aniline and toluidine may be lost in steam during hydrolysis, even from acid solution. Finally, there is the complication of the choice of a suitable standard since hydrolysis of the urines may yield more than one diazotizable compound, and the diazo colours given by the compounds differ considerably, e.g. aniline and *p*-aminophenol; toluidine, aminocresol and aminobenzoic acid. The standards used for the diazo estimations, which were performed, were aniline sulphate for phenylurea, *o*-toluidine hydrochloride for *o*-tolylurea and *m*- and *p*-aminobenzoic acids for the corresponding tolylureas. An attempt was made to devise a method capable of distinguishing between acetamido and ureido compounds, but it was found that even under the mildest conditions necessary for the hydrolysis of the former, the ureido derivatives were hydrolyzed to some extent.

## RESULTS

### *Quantitative studies*

*Excretion of normal metabolites.* The average daily excretion of normal metabolites by individual rabbits used in this study was as follows: ether-soluble acid (as hippuric acid), 634–853 mg., the day-to-day variation for each rabbit usually being less than $\pm 7\%$ of the mean; reducing material (as glucuronic acid), 110–202 mg., the day-to-day variation for each rabbit usually being less than $\pm 13\%$ of the mean; average increase of reducing material on hydrolysis, 76%; ethereal sulphate, 32–63 mg. $SO_3$, the day-to-day variation for each rabbit usually being within $\pm 10\%$ (i.e. approx. 5 mg. $SO_3$) of the mean.

*Metabolism of* N*-phenylurea.* Table 2 summarizes the results obtained. It can be seen that an average of 46% of the dose is hydroxylated and conjugated with sulphuric acid (20%) and glucuronic acid (26%). The principal hydroxylation product is *N*-(*p*-hydroxyphenyl)urea and some, at least, is excreted unconjugated, as evidenced by the purple colour which phenylurea urines give with $FeCl_3$. The remainder of the dose is presumably excreted unchanged, but it was not possible to demonstrate this quantitatively, owing to the stability of the ureido group.

*Metabolism of* o*-tolylurea.* The quantitative results obtained are given in Table 3. Little or no oxidation of the methyl group occurs, since there is no increase in excretion of ether-soluble acid or ester glucuronide, but an average of 35% of the dose is hydroxylated, about 4% of the resulting *N*-(4-hydroxy-2-methylphenyl)urea being conjugated with sulphuric acid and 31% with glucuronic acid.

*Metabolism of* m*-tolylurea.* As may be seen from Table 4, which contains the quantitative results obtained, an average of 45% of the dose is oxidized to a carboxylic acid, of which the main part (41%) is excreted unconjugated and only 4% as ester glucuronide. In addition hydroxylation takes place (14%), the product being excreted conjugated as both ethereal sulphate (10%) and ether glucuronide (14%). The reducing properties of the hydroxylation product (*N*-(4-hydroxy-3-methylphenyl)urea) under the conditions of the estimation were allowed for as previously described.

*Metabolism of* p*-tolylurea.* Table 5 summarizes the results obtained. About 80% of the dose is oxidized to *p*-ureidobenzoic acid, of which only a small amount (6%) is excreted in conjugated form.

*Stability of ureido compounds to rabbit-liver extracts.* The experiments described above do not preclude the possibility that slight hydrolysis of the ureido group occurs *in vivo*. If this does occur it might be expected that extracts of rabbit liver would bring about the hydrolysis *in vitro*. It was, however, found that incubation of *N*-phenylurea, *N*-(*p*-hydroxyphenyl)urea, the three tolylureas and *m*- and *p*-ureidobenzoic acids (*c.* 0·01 M in buffer pH 7·4) with rabbit-liver extract prepared as previously described (Bray, James, Ryman & Thorpe, 1948*a*) gave rise to no liberation of diazotizable material beyond that formed by spontaneous hydrolysis in the controls. Similarly, *N*-phenylurea was not hydrolyzed by rabbit-kidney extract.

### *Qualitative studies*

N*-Phenylurea.* The urine excreted by rabbits which had received phenylurea gave a purple colour with $FeCl_3$, suggesting that an unconjugated phenolic compound was excreted. We were not able to isolate the compound responsible, however, by means of ether extraction at various values of pH. The quantitative results given in Table 2 suggest that about half the dose might be excreted unchanged, but it was not possible to isolate *N*-phenylurea from the urine by extraction with either ether or methyl ethyl ketone. (The latter extracts *N*-phenylurea which has been added to urine.) A small amount of phenylurea was obtained during the isolation of the glucuronide (*q.v.*), but

Table 2. *Metabolism of* N*-phenylurea in the rabbit*

| Rabbit no. | Dose (g./kg.) | Percentage of dose excreted as: Ether glucuronide | Free amino compounds | Ethereal sulphate |
|---|---|---|---|---|
| 152 | 0·3 | 21 | 0·7 | 25 |
| | | 17 | <1·0 | 24 |
| | | 24 | 0 | — |
| 156 | 0·3 | 31 | 2·4 | 16 |
| | | 35 | <1·0 | — |
| | | 33 | 0 | — |
| 160 | 0·3 | 25 | 0 | 15 |
| | | 12 | <1·0 | — |
| | | 32 | 0 | — |
| | Av. | **26** | **<1·0** | **20** |

it was not possible to obtain an amount approximating to that unaccounted for.

Phenylurea urine was hydrolyzed with acid (cf. Bray & Thorpe, 1948), neutralized and continuously extracted with ether. From 9·0 g. *N*-phenylurea given to ten rabbits 0·6 g. of a crystalline compound, m.p. 186°, was obtained. It gave a positive diazo reaction and a purple-brown colour with $FeCl_3$. Mixed with an authentic specimen of *p*-aminophenol (m.p. 187°) it melted at 187°. The identity of the compound was further confirmed by comparing its *N*-acetyl derivative (m.p. 168°) with authentic *p*-acetamidophenol (m.p. 169°). The mixed m.p. was 169°. In the diazo tests on the crude extract a yellow colour was obtained with $HNO_2$; it was thought that this might be due to the presence of an *o*-aminophenol derivative, but attempts at its isolation were unsuccessful.

From 6 g. phenylurea given to six rabbits a glucuronide was isolated as previously described (Bray *et al.* 1947) from which a crystalline potassium salt (150 mg.) was obtained, as described by Morinaka (1922) and Tsunoo (1935). The properties are given and compared in Table 6 with those of the compounds obtained by the Japanese workers. We were unable to isolate *N*-(*p*-hydroxyphenyl)urea by the hydrolysis of the glucuronide, but on sublimation of the potassium salt with soda lime, *p*-aminophenol was obtained and identified as previously described. The glucuronide did not form a crystalline barium salt. During an attempt to isolate the glucuronide through its barium salt a small amount of *N*-phenylurea was isolated.

*o-Tolylurea.* Extraction of neutral *o*-tolylurea urine with ether gave a small quantity of crystals, m.p. 190°, which were identified as *o*-tolylurea. Yield, 8% of the dose. The

Table 3. *Metabolism of* o-*tolylurea in the rabbit*

| Rabbit no. | Dose (g./kg.) | Percentage of dose excreted as: Ether-soluble acid | Ester glucuronide | Ether glucuronide | Free amino compounds | Ethereal sulphate |
|---|---|---|---|---|---|---|
| 152 | 0·3 | 0 | 0 | 28 | <1·0 | 6 |
| | | 0 | 0 | 10 | — | — |
| | | 0 | 0 | 28 | 1·4 | — |
| | | 0 | 0 | 38 | <1·0 | — |
| 156 | 0·3 | 0 | 0 | 33 | <1·0 | — |
| | | 15 | 0 | 17 | — | 0 |
| | | 0 | 0 | 37 | 1·8 | — |
| | | 0 | 0 | 32 | <1·0 | — |
| 166 | 0·3 | 0 | 0 | 28 | <1·0 | — |
| | | 0 | 0 | 19 | — | — |
| | | 0 | 0 | 44 | <1·0 | 5 |
| | | 0 | 0 | 30 | <1·0 | — |
| 168 | 0·3 | 12 | 0 | 56 | <1·0 | — |
| | | 27 | 0 | 20 | — | — |
| | | 0 | 0 | 31 | 1·7 | 3 |
| | | 8 | 0 | 37 | <1·0 | — |
| | Av. | 4* | 0 | 31 | <1·0 | 4 |

* Since there is no other evidence of oxidation of the methyl group it seems likely that the positive values in this column are due to abnormal fluctuations in the base-line values, and that the true figure here is 0.

Table 4. *Metabolism of* m-*tolylurea in the rabbit*

| Rabbit no. | Dose (g./kg.) | Percentage of dose excreted as: Ether-soluble acid | Ester glucuronide | Ether glucuronide | Free amino compounds | Ethereal sulphate |
|---|---|---|---|---|---|---|
| 178 | 0·3 | 44 | 7 | 11 | 1·4 | 12 |
| | | 46 | 4 | 12 | 1·8 | — |
| | | 27 | 0 | 13 | 1·1 | — |
| | | 40 | 3 | 13 | — | — |
| 182 | 0·3 | 42 | 7 | 12 | 1·3 | — |
| | | 31 | 0 | 18 | 2·6 | 8 |
| | | 36 | 6 | 9 | 1·6 | — |
| | | 36 | 4 | 21 | — | — |
| 190 | 0·3 | 46 | 4 | 15 | 1·5 | — |
| | | 41 | 3 | 17 | 2·2 | — |
| | | 49 | 4 | 8 | 1·5 | 11 |
| | | 40 | 3 | 19 | — | — |
| 191 | 0·3 | 52 | 9 | 14 | 1·4 | — |
| | | 48 | 5 | 14 | 1·8 | — |
| | | 36 | 4 | 6 | 1·5 | — |
| | | 41 | 5 | 20 | — | 9 |
| | Av. | 41 | 4 | 14 | 1·5 | 10 |

Table 5. *Metabolism of p-tolylurea in the rabbit*

| Rabbit no. | Dose (g./kg.) | Percentage of dose excreted as: Ether-soluble acid | Ester glucuronide | Ether glucuronide | Free amino compounds | Ethereal sulphate |
|---|---|---|---|---|---|---|
| 152 | 0·3 | 52 | 2 | 0 | 2·3 | 0 |
| | | 81 | 4 | 0 | 1·7 | — |
| | | 65 | 4 | 0 | 2·2 | — |
| | | 88 | — | — | — | — |
| 156 | 0·3 | 85 | 5 | 0 | 2·1 | 1 |
| | | 78 | 8 | 0 | 2·8 | — |
| | | 79 | 8 | 0 | 2·1 | — |
| | | 85 | — | — | — | — |
| 160 | 0·3 | — | 8 | 0 | 2·9 | — |
| | | — | 7 | 0 | 1·7 | — |
| | | — | 5 | 0 | 1·8 | 0 |
| 168 | 0·3 | 96 | — | — | — | — |
| | | 80 | — | — | — | — |
| | | 87 | — | — | — | — |
| | Av. | **80** | **6** | **0** | **2·2** | **0** |

Table 6. *Properties of potassium salts of 4-ureidophenylglucuronide*

| | From *N*-phenylurea in rabbit | From 'Elbon'* in rabbit | From 'Ethynal'†: In dog | From 'Ethynal'†: In rabbit | Calc. for $C_{13}H_{15}N_2O_8K$ |
|---|---|---|---|---|---|
| % N | 7·69 | 7·59 | 7·5 | 7·31 | 7·65 |
| % K | 10·70 | 10·62 | 11·07 | — | 10·68 |
| $[\alpha]_D$ in water (°) | −70·1 at 23 | −74·99 at 20 | −67·8 at 29 | −59·3 at 23 | — |
| M.p. (decomp.) (°) | 257 | 231 | 243 | 231 | — |

* Morinaka (1922). † Tsunoo (1935).

urine was acidified and re-extracted, but only hippuric acid was isolated. No acetotoluidide was detected.

Extraction of neutral, hydrolyzed urine gave a crystalline product which darkened rapidly. Treatment with charcoal and recrystallization from aqueous ethanol containing $SO_2$ gave 50 mg. light yellow crystals, m.p. 178°, which gave a red-purple coloration with $FeCl_3$ and positive diazo and indophenol reactions: mixed with an authentic specimen of 2-amino-5-hydroxytoluene (m.p. 179°), m.p. 179°. The acetate of the compound isolated (m.p. 125°) gave a blue-violet colour with $FeCl_3$ and was shown to be identical with an authentic specimen of 2-acetamido-5-hydroxytoluene.

Crystalline barium or potassium salts of the glucuronide could not be obtained and attempts to obtain a crystalline glucuronide were unsuccessful, the product being a syrup. On hydrolysis of this with 2N-HCl 50 mg. of crystals, m.p. 230°, were obtained. The identity of this compound with *N*-(4-hydroxy-2-methylphenyl)urea was shown by comparison with an authentic specimen. (Found: N, 16·6. Calc. for $C_8H_{10}N_2O_2$: N, 16·89%.)

m-*Tolylurea*. Ether extraction of *m*-tolylurea urine as collected yielded no product, but from the acidified (pH 1·5) urine a compound, m.p. 290° (decomp.), was obtained in a yield corresponding to 25% of the dose administered. The compound had the same properties as synthetic *m*-ureidobenzoic acid and contained 15·6% N; calc. for $C_8H_8N_2O_3$: N, 15·56%. After hydrolysis for 2 hr. with 2N-HCl, extraction with ether gave a crystalline product, m.p. 177°, which was shown to be identical with authentic *m*-aminobenzoic acid, and its identity was further confirmed by comparison of the *N*-acetyl derivatives. It is, therefore, concluded that the compound originally isolated was *m*-ureidobenzoic acid. No *m*-acetotoluidide or toluidine was isolated.

Ether extraction of neutral, hydrolyzed *m*-tolylurea urine yielded yellow-brown crystals, m.p. 174°, which gave with $FeCl_3$ a red-brown colour, changing slowly to red-purple, and a positive indophenol reaction. On diazotizing and coupling with *N*-(1-naphthyl)ethylenediamine dihydrochloride a pale blue colour slowly giving place to purple was observed, which is characteristic of some aminocresols, e.g. 5-amino-2-hydroxytoluene, 2-amino-5-hydroxytoluene. Yield, 40 mg. from four doses of 0·9 g. This compound was identical with an authentic specimen of 5-amino-2-hydroxytoluene (m.p. 175°), mixed m.p. 174°. Its acetyl derivative melted at 180°, and did not depress the melting point of a synthetic specimen of 5-acetamido-2-hydroxytoluene. This does not give a colour with $FeCl_3$. (Found: N, 8·31. Calc. for $C_9H_{11}NO_2$: N, 8·48%.)

Attempts to obtain a crystalline glucuronide (or salt) were unsuccessful, only a syrup being obtained. The syrup was hydrolyzed by refluxing for 2 hr. with 2N-HCl and extracted with ether. The ether-soluble material was divided into two parts. From one 5-amino-2-hydroxytoluene was obtained and identified as above: the other part was acetylated, giving 5-acetamido-2-hydroxytoluene and *m*-acetamidobenzoic acid. This finding is in agreement with the results shown in Table 4, indicating the presence of both ester and ether type glucuronides. After several attempts to obtain *N*-(4-hydroxy-3-methylphenyl)urea by milder hydrolysis of the glucuronide syrup (20 min. on boiling water bath with N-HCl), about 5 mg. straw-coloured needles, m.p. 195°, were obtained in one experiment. These gave a brown colour with $FeCl_3$ and, after hydrolysis, indophenol

and diazo reactions similar to those given by 5-amino-2-hydroxytoluene. Mixed m.p. with authentic *N*-(4-hydroxy-3-methylphenyl)urea (m.p. 198°), 195°. The amount was insufficient for purification for analysis. Small amounts of *m*-tolylurea were also obtained.

*p-Tolylurea.* Extraction of acidified *p*-tolylurea urine yielded an acid which did not melt below 300°. Yield 1·2 g. from a total dose of 3·6 g. After recrystallization from aqueous ethanol it was found to contain 16·0% N. (Calc. for $C_8H_8N_2O_3$: N, 15·56%.) Acid hydrolysis gave a crystalline product, m.p. 187°, which was shown to be identical with *p*-aminobenzoic acid (m.p. 189°); mixed m.p. 189°. This was confirmed by comparison of the acetamido compounds. It is, therefore, concluded that the product isolated from urine was *p*-ureidobenzoic acid. No other metabolite was isolated.

## DISCUSSION

Table 7 summarizes the quantitative results obtained in this investigation, together with the corresponding results obtained with acetotoluidides (Bray & Thorpe, 1948). It can be seen that the metabolic fates of the two types of compound are very similar, the main differences being quantitative rather than qualitative, e.g. the percentage conjugation of the hydroxylation products with sulphuric and glucuronic acids.

Although the ureido group in the compounds studied appears to be stable *in vivo*, as evidenced by the fact that various metabolites were isolated with it intact, the tendency to spontaneous decomposition suggests that some hydrolysis might be expected to occur. It is theoretically possible that amino compounds arising in this way may be acetylated before excretion. Attempts were made to identify acetamidobenzoic acids and acetotoluidides among the excretion products, in every case without success. Owing to the difficulties of distinguishing between and separating *N*-phenylurea derivatives and acetamido compounds, we do not regard the question as having been finally settled, although enzyme experiments lend support to the view that the ureido group is stable *in vivo*.

Other compounds in which a ureido group is attached directly to the aromatic ring do not appear to have been investigated, but the literature provides some information as to the stability of this group attached to aliphatic or other residues. Koehne (1895) stated that biuret was excreted unchanged by the dog, and Gaebler & Keltch (1926) found over 50% of hydantoin or hydantoic acid was excreted by the dog as hydantoic acid, there being no evidence of further breakdown. Several diphenylhydantoins, however, are excreted by man and dog as the diphenylhydantoic acids, and, to a much greater extent, as the corresponding α-aminodiphenylacetic acids (Hine & Kozelka, 1943). Benzoylurea (Koehne, 1895) and benzylidenediureide (Bülow, 1894) in the dog appear to lose the ureido group intact with the formation of urea.

The hydroxylation products of the tolylureas resemble exactly those from the acetotoluidides, all their constitutions being in agreement with the general rule suggested previously (Bray *et al.* 1948*b*). It is clear, therefore, that modification of the aromatic amino group by conversion to a ureido group does not change the nature of its influence on the position taken up by the entering hydroxyl group.

## SUMMARY

1. The metabolism of the three *N*-tolylureas in the rabbit has been shown to be similar to that of the corresponding acetotoluidides.

2. The ureido group in these compounds appears to be stable in the rabbit and to rabbit-liver extracts *in vitro*.

3. *o*-Tolylurea is converted to *N*-(4-hydroxy-2-methylphenyl)urea (35% of the dose) which is excreted as ethereal sulphate (4%) and glucuronide (31%). The methyl group is not oxidized to any

Table 7. *Comparison of metabolism of* N*-tolylureas, acetotoluidides and* N*-phenylurea in the rabbit*

| Compound | Percentage of compound: Excreted as ether-soluble acid | Excreted as ester glucuronide | Oxidized to carboxylic acid | Excreted as ether glucuronide | Excreted as ethereal sulphate | Hydroxylated | Excreted with free amino group§ |
|---|---|---|---|---|---|---|---|
| *N-o*-Tolylurea | 0 | 0 | 0 | 31 | 4 | 35 | <1·0 |
| *o*-Acetotoluidide* | 8 | — | 6† | — | 32 | 32‡ | 5·6 |
| *N-m*-Tolylurea | 41 | 4 | 45 | 14 | 10 | 24 | 1·5 |
| *m*-Acetotoluidide* | 34 | — | 25† | — | 10 | 10‡ | Trace |
| *N-p*-Tolylurea | 80 | 6 | 86 | 0 | 0 | 0 | 2·2 |
| *p*-Acetotoluidide* | 78 | 10 | 96† | — | 0 | 0‡ | 0·8 |
| *N*-Phenylurea | — | 0 | — | 26 | 20 | 46 | <1·0 |

* Bray & Thorpe (1948).

† From total diazotizable material.

‡ May be low since no ether glucuronide value is included.

§ All the values recorded in this column, with the exception of that for *o*-acetotoluidide, are probably due to spontaneous hydrolysis. The acetotoluidides hydrolyze spontaneously, although to a smaller extent than does *N*-phenylurea (Bray, Lake & Thorpe, unpublished).

appreciable extent. Some *o*-tolylurea is excreted unchanged.

4. *m*-Tolylurea is oxidized to *m*-ureidobenzoic acid (45%), 4% of which is excreted as ester glucuronide, and hydroxylated to *N*-(4-hydroxy-3-methylphenyl)urea (24%), of which 10% is excreted as ethereal sulphate and 14% as ether glucuronide. Some *m*-tolylurea is excreted unchanged.

5. *p*-Tolylurea is almost completely oxidized to *p*-ureidobenzoic acid (86%), of which only 6% is conjugated as ester glucuronide, the remainder being excreted free.

6. *N*-Phenylurea is partly hydroxylated to *N*-(*p*-hydroxyphenyl)urea (46%) which is conjugated almost equally with sulphuric acid (20%) and glucuronic acid (26%). Some *N*-phenylurea is excreted unchanged. 4-Ureidophenylglucuronide was isolated from *N*-phenylurea urine as its crystalline potassium salt.

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# The Effect of Deamination and Esterification on the Reactivity of Collagen

By JOANE H. BOWES and R. H. KENTEN
*The British Leather Manufacturers' Research Association, London, S.E.* 1

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The most important reactive groups in collagen are amino, guanidino and carboxyl, all of which are present in comparatively large numbers; few phenolic, hydroxyl and imidazole groups are present and indole groups are absent (Bowes & Kenten, 1948*a*). A study has now been made of the effect of modification of the amino, guanidino and carboxyl groups on the acid- and base-binding capacity, and on the swelling of collagen. The combination of the modified collagens with vegetable tannins, basic chromium salts and formaldehyde has also been investigated primarily because of the importance of these three classes of compound in the conversion of collagen to leather. Moreover, the fixation of metals by proteins is of general interest, and information concerning the factors affecting the fixation of tannins may help to throw light on the association of tannins and proteins in nature.

The amino groups of proteins can readily be converted to hydroxyl groups by the action of nitrous acid. The reaction is not entirely specific, however, since at low pH values, or if the reaction is prolonged, modification of the guanidino, imidazole, tyrosine and glutamide groups may also take place (for review of literature see Olcott & Fraenkel-Conrat, 1947). With soluble proteins, reaction with the amino groups is rapid and other reactions are probably negligible, but with fibrous proteins, such

as collagen, the reaction must be prolonged to allow time for the nitrous acid to diffuse into the interior of the fibres, and some modification of other groups probably occurs. Previous workers who have used this method with collagen did not determine the extent of specificity of the reaction (Thomas & Foster, 1926; Thomas & Kelly, 1926; Gustavson, 1926; Meunier & Schweikert, 1935; Atkin, 1937; Bowes & Pleass, 1939; Highberger & Retzsch, 1939; Wilson & Yu, 1941; Gustavson, 1943; Lollar, 1943; Chang, Yen & Chen, 1944; Theis, 1945).

Modification of the guanidino groups has received little attention and no satisfactory method appears to be available. Treatment with hypochlorite, as in the Sakaguchi reaction, has been found to destroy all the arginine present in soluble proteins, but histidine, tyrosine, tryptophan and lysine are also affected, though to a lesser extent (Sakaguchi, 1925). Only about 43% of the arginine in collagen is affected by the reaction (Highberger & Salcedo, 1940). In the present investigation, it was again found that only 40–50% of the arginine in collagen was destroyed by this treatment. Roche & Morgue (1946) have shown that the reactivity, in the Sakaguchi reaction, of the guanidino group in amino-acid derivatives is influenced by the presence of other groups, some of which, notably aliphatic hydroxyl, prevent over 90% of the guanidino groups from reacting. It is possible that the difficulty of removing more than about half the guanidino groups from the collagen is related to the same cause. Since the reaction was obviously not specific to the guanidino groups, and was accompanied by extensive general breakdown of the collagen, it was not studied in detail.

A number of the reagents which react with the carboxyl groups of proteins, e.g. diazomethane, acetic anhydride, keten, methanol, are not specific to carboxyl groups (Herriott, 1947; Olcott & Fraenkel-Conrat, 1947), and with some the conditions necessary for reaction cause breakdown of the protein structure (Fodor & Epstein, 1937). Blackburn, Carter & Phillips (1941) and Blackburn & Phillips (1944) have studied the methylation of wool with methyl sulphate and methyl halides, and conclude that the main reaction is esterification of the carboxyl groups. It appeared, therefore, that these methods were likely to be satisfactory for modifying the carboxyl groups of collagen without affecting other groups to any appreciable extent, and with minimum risk of degradation.

## EXPERIMENTAL METHODS

### *Material*

The collagen was prepared from sheepskins which had been dewooled with $Ca(OH)_2$ and $Na_2S$ ('limed'), treated with pancreatic trypsin ('bated'), and preserved with $H_2SO_4$ and NaCl ('pickled'). The skins were washed free of acid and salt, dehydrated with acetone, cut into strips $1 \times 5$ cm. (the edges of the skins being discarded), brought to equilibrium with distilled water (pH *c.* 5), and finally dehydrated with acetone. The titration curves of three batches of collagen prepared as above (*A*, *B* and *C*) are given in a previous paper (Bowes & Kenten, 1948*b*); they are referred to in text and tables as collagen *A*, *B* and *C*, with the prefixes *D* and *M* to denote deaminated and methylated, respectively.

### *Treatment of collagen*

*Deamination.* The method used was based on that of Thomas & Foster (1926). Collagen (100 g.) was soaked in distilled water overnight, 100 g. $NaNO_2$ then added, followed by 100 ml. glacial acetic acid. A stream of $CO_2$ was passed through the solution to minimize oxidation of nitrous to nitric acid and to agitate the solution. After 24 hr., a further 100 g. $NaNO_2$ and 100 ml. acetic acid were added and the reaction allowed to proceed for another 24 hr. The collagen was rinsed with water, washed to remove acid in several changes of water containing 10% (w/v) NaCl to minimize swelling, brought to equilibrium with distilled water (pH 5·0), and dehydrated with acetone. Two batches of collagen *C* ($DC_1$ and $DC_2$) and one of collagen *A* (*DA*) were treated in this way.

*Methylation.* Methods used were similar to those of Blackburn *et al.* (1941): (1) Dimethyl sulphate. Collagen (50 g.) was shaken with 500 ml. M-Na acetate buffer (pH 7·5) and 25 ml. methyl sulphate for 1 hr. This procedure was repeated fourteen or more times. To reduce the extreme swelling of the methylated collagen it was placed in a 10% (w/v) solution of NaCl for 20 hr., then washed in distilled water until free from $Cl^-$, and dehydrated with acetone. (2) Methyl bromide. The collagen was brought to equilibrium with a borate buffer at pH 9·0, dehydrated with acetone, air dried, and immersed in methyl bromide for 14 days. The latter was then allowed to evaporate, and the collagen washed as before and dehydrated with acetone. In some cases the collagen was rebuffered and treated for a further 14 days with methyl bromide. Several batches of collagen *A* ($MA_1$ to $MA_4$) and of collagen *C* ($MC_1$ to $MC_5$) were methylated; also the oxhide collagen prepared as previously described (Bowes & Kenten, 1948*a*).

*Combination of modified collagen with tannic acid, mimosa tannins, chromium and formaldehyde.* The modified collagen (2 g.) in the form of pieces 1 cm. square, and a piece $1 \times 5$ cm. for shrinkage-temperature determinations were soaked in water overnight and then placed in 100 ml. of the following tanning solutions adjusted to different pH values for the times stated. Approximate equilibrium should have been reached in these times:

(1) 10% (w/v) tannic acid (British Drug Houses, Ltd.) for 7 days.
(2) 10% (w/v) tannic acid + 0·5 M-NaCl for 7 days.
(3) 10% (w/v) commercial mimosa tannin extract for 7 days.
(4) 3·4% (w/v) chromic sulphate for 4 days.
(5) 0·36% (w/v) formaldehyde for 3 days.

All pH values were attained with HCl or NaOH. The tannin solutions were initially adjusted to the required pH and then subsequently twice daily during the treatment. The pH of the $Cr_2(SO_4)_3$ solutions was adjusted after 24 hr., and maintained at this pH value by frequent further additions

during the following 48 hr.; no additions were made in the last 24 hr. and the pH fell slightly during this time. The pH's of the formaldehyde solutions were initially adjusted, and then allowed to fall during the treatment.

On the completion of the treatment the pieces were drained, washed in Wilson-Kern extractors (Wilson & Kern, 1921) with 9 l. distilled water for 20 hr. and air dried.

### *Analysis of products*

*Total* N, *amide* N, *amino* N *and arginine*. The methods have been described previously (Bowes & Kenten, 1948*a*, *b*).

*Lysine*. This was determined using a specific decarboxylase (Gale, 1945). Though this method may also determine an unknown fraction of the hydroxylysine it gives an indication of the extent to which ε-amino groups are modified. Recent evidence (Heathcote, 1948) suggests that the apparent reaction of hydroxylysine with lysine decarboxylase may in fact be due to contamination of the hydroxylysine with lysine.

O-*Methyl groups*. These were determined as methyl iodide using a semimicroalkoxyl apparatus (Clark, 1932, 1939; Viebock & Schwappach, 1930). Methionine also yields methyl iodide by this method (Baernstein, 1932; 1936): the oxhide and sheepskin collagens gave values of 0·08 and 0·05% of methyl, respectively.

N-*Methyl groups*. The method is described by Pregl (1930). *O*-Methyl and *N*-methyl contents are expressed as a percentage of methyl on moisture- and ash-free collagen and are corrected for the methionine content of the collagen.

*Chloride and sulphate*. The former was determined as described by Highberger & Moore (1929). In the determination of $SO_4^{=}$, methylated collagen (3 g.) was heated for 3 hr. on a steam bath with 30 ml. 2N-HCl. The solution was evaporated to dryness, the residue dissolved in 100 ml. hot water and boiled with charcoal (Norite) for 15 min. The solution was filtered, the charcoal extracted with boiling water, and the combined filtrates neutralized with 2 ml. 5N-HCl, and the $SO_4^{=}$ determined as $BaSO_4$. Collagen gave no $SO_4^{=}$ by this method, and recovery tests showed an accuracy of ±5%.

*Tannin in combination with collagen*. This was determined by drying in a vacuum oven at 100–102° for 6 hr. and weighing. The amount of tannin fixed/100 g. moisture- and ash-free collagen was then calculated from the increase in weight of the collagen.

*Chromium*. The method used is described by Davies & Innes (1944). Results are expressed as g. $Cr_2O_3$/100 ml. and mmol. Cr/g. moisture- and ash-free collagen.

*Formaldehyde*. The treated collagen (2 g.) was steam-distilled with 40 ml. 2N-$H_2SO_4$, 500 ml. distillate being collected. The formaldehyde was precipitated from suitable samples of distillate with dimedone (Yoe & Reid, 1941).

*Shrinkage temperature*. The temperature at which shrinkage occurs in water is a property characteristic of both collagen and treated collagens. It has been related to cross linking in the protein structure (Theis, 1946). A modified form of the apparatus described by Küntzel (1943) was used, in which a stream of air was blown continuously round the side arm to aid circulation. When the shrinkage temperature exceeded 100° the tanned collagens were wetted by immersion in water under reduced pressure and the determination then made in liquid paraffin. The shrinkage temperature so determined is the same as that determined in water under pressure.

*Titration and swelling curves*. These were determined as previously described (Bowes & Kenten, 1948*a*, *b*).

## RESULTS

Except where otherwise stated, results are expressed on a moisture- and ash-free basis. Amino-N contents, etc., are expressed as mmol./g. rather than as a percentage of total N, since variations in the N content of the modified collagens render the latter method of expression unsuitable for comparative purposes.

### *Analysis*

*Deamination*. The deaminated collagens had the characteristic orange-yellow colour associated with proteins which have been treated with nitrous acid (cf. Philpot & Small, 1938). Reaction of the amino groups was almost complete in all the samples examined (Table 1). As with other proteins (Steudel & Schumann, 1929; Wiley & Lewis, 1930) there was some loss of arginine (*c.* 20%).

The amide N was higher than that of the original collagen, and this additional $NH_3$ may have come from —NH.CN groups formed by the action of the nitrous acid on guanidino groups (see p. 149). The increase in amide N was approximately equal to the decrease in arginine content.

*Methylation*. The methylated collagens varied from brown to grey in colour and in two cases were almost black. They had a smell of methylamines. The methoxyl content of the different collagens treated with methyl sulphate varied from 0·94 to 1·57% (as methyl), the maximum methoxyl content being attained in 14 methylations (see oxhide collagen, Table 2). Variations in methoxyl content are probably due to slow hydrolysis of these groups during washing (see below). Methylation with methyl bromide introduced rather fewer methoxyl groups. Deamination of the collagen did not affect the extent of methylation with methyl sulphate, but increased the methoxyl groups introduced by methyl bromide to the same value as that obtained with methyl sulphate. This suggests that the lower values obtained with methyl bromide on untreated collagen are due, not to inability to introduce the same number of methoxyl groups as methyl sulphate, but to the difficulty of keeping the collagen at a suitable pH for the reaction to proceed to completion; diminution in the number of basic groups will reduce the change in pH of the collagen which occurs as a result of methylation of the carboxyl groups, and hence makes it easier to keep the collagen at the required pH.

Methylated collagen fixes $SO_4^{=}$ and $Cl^{-}$ approximately equivalent to the methoxyl groups introduced. Blackburn *et al.* (1941) found that when wool was methylated with

Table 1. *Analyses of collagens and deaminated collagens*

(Results expressed on moisture- and ash-free basis except where otherwise stated.)

| | Untreated | Treated with $HNO_2$ | | Untreated | Treated with $HNO_2$ |
|---|---|---|---|---|---|
| | *C* | $DC_1$ | $DC_2$ | *A* | $DA_1$ |
| Moisture (%) air dry | 16·2 | 6·3* | 16·0 | 18·1 | 14·1 |
| Ash (%) air dry | 0·23 | 1·78 | 0·23 | 0·14 | 0·58 |
| Total N (%) | 17·2 | 16·9 | — | 17·3 | 17·3 |
| Amide N (mmol./g.) | 0·25 | 0·38 | 0·39 | 0·30 | 0·38 |
| Amino N (mmol./g.) | 0·36 | 0·05 | — | — | 0·04 |
| Lysine† (mmol./g.) | 0·30 | — | Nil | 0·31 | 0·01 |
| Arginine (mmol./g.) | 0·44 | — | 0·35 | 0·43 | 0·37 |

* The low value is due to the collagen having been dried in a desiccator over anhydrous $CaCl_2$ for some days; other samples air-dry.

† May include some hydroxylysine.

Table 2. *The methylation of collagen and deaminated collagen with methyl sulphate and with methyl bromide*

| Sample | $OCH_3$ groups introduced (as $CH_3$) (%) | $OCH_3$ groups introduced (as $CH_3$) (mmol./g.) | Anions (m-equiv./g. collagen) Chloride* | Sulphate | Total |
|---|---|---|---|---|---|
| **Methylated with methyl sulphate** | | | | | |
| Oxhide collagen (free carboxyl groups, 0·87 mmol./g.): | | | | | |
| Methylated 14 times $MN_1$ | 1·19 | 0·79 | Nil | 0·64 | 0·64 |
| Methylated 14 times $MN_2$ | 0·94 | 0·63 | Nil | 0·52 | 0·52 |
| Methylated 16 times $MN_3$ | 1·37 | 0·91 | Nil | 0·78 | 0·78 |
| Methylated 16 times $MN_4$ | 1·22 | 0·81 | Nil | 0·70 | 0·70 |
| Methylated 18 times $MN_5$ | 1·15 | 0·77 | Nil | 0·65 | 0·65 |
| Methylated 18 times $MN_6$ | 1·24 | 0·83 | Nil | 0·75 | 0·75 |
| Collagen *A* (free carboxyl groups 0·96 mmol./g.): | | | | | |
| $MA_1$ | 1·25 | 0·83 | 0·61 | — | — |
| $MA_2$ | 1·32 | 0·88 | 0·73 | 0·07 | 0·80 |
| $MA_3$ | 1·49 | 0·99 | 0·58 | 0·40 | 0·98 |
| $MA_4$ | 1·32 | 0·88 | 0·50 | 0·34 | 0·84 |
| Collagen *C* (free carboxyl groups, 1·01 mmol./g.): | | | | | |
| $MC_1$ | 1·32 | 0·88 | — | — | — |
| $MC_2$ | 1·57 | 1·05 | 0·52 | 0·52 | 1·04 |
| $MC_3$ | 1·18 | 0·79 | 0·65 | — | — |
| $MC_4$ | 1·04 | 0·69 | — | — | — |
| Collagen *A* after treatment with nitrous acid: | | | | | |
| $MDA_1$ | 1·32 | 0·88 | 0·42 | 0·05 | 0·47 |
| **Methylated with methyl bromide** | | | | | |
| Collagen *C*: | | | | | |
| Methylated 14 days $MC_5$ | 0·35 | 0·23 | 0·11 | — | 0·11 |
| Methylated 28 days $MC_6$ | 0·85 | 0·57 | 0·48 | — | 0·48 |
| Collagen *C* after treatment with nitrous acid: | | | | | |
| Methylated 28 days $MDC_1$ | 1·22 | 0·82 | 0·30 | — | 0·30 |

* Arises from displacement of $SO_4^{=}$ by $Cl^-$ during immersion of methylated collagen in NaCl.

methyl iodide and bromide, $I^-$ and $Br^-$ were held in excess of the methoxyl groups, and attributed this to *N*-methylation. Attempts to determine the percentage of *N*-methyl groups were unsuccessful. Under the conditions of Pregl's method proteins and amino-acids yield appreciable amounts of volatile iodides, and large and variable values for the apparent *N*-methyl are obtained (Burns, 1914; Geake & Nierenstein, 1914; Lindley & Phillips, 1947). Determination of *N*-methyl groups in methylated collagen gave values of the same order as those obtained with the original collagen. Though it may be unwise to conclude that no *N*-methylation takes place, it appears that it only occurs to a small extent, if at all. The amino N of the collagen methylated with methyl bromide ($MC_5$) and with methyl sulphate ($MC_4$) was 0·24 and 0·31 mmol./g., respectively, compared with a value of 0·36 mmol./g. for the original collagen. The amino N of collagen $MA_4$ methylated with methyl sulphate was 0·30 mmol./g.

*Stability.* Methoxyl groups were only slowly split by hydrolysis when the methylated collagens were immersed in buffer solutions between pH 2 and 8, but outside this range the stability decreased rapidly with rise or fall in pH (see Fig. 1). A similar stability curve for methylated wool constructed from the data of Blackburn *et al.* (1941) is given for comparison. Soaking in water at pH 5·0 for 20 days

reduced the methoxyl content from 1·38 to 1·12% (as methyl).

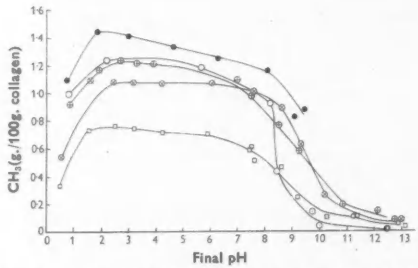


Fig. 1. Stability of $OCH_3$ groups introduced into collagen, deaminated collagen and wool in pH range 0·5–13·0. Percentage $CH_3$ after soaking in universal buffer solution for 2·5 days. ⊙—⊙, collagen esterified with methyl sulphate; ⊡—⊡, collagen esterified with methyl bromide; ⊕—⊕, collagen deaminated and esterified with methyl sulphate; ⊗—⊗, collagen deaminated and esterified with methyl bromide; •—•, wool esterified with methyl sulphate (from Blackburn *et al.* 1941).

Table 3. *Soluble nitrogen liberated from methylated collagen after* 3 *days' exposure to alkaline solutions*

| Sample | pH of solution | Soluble N (mg./g.) | $OCH_3$ groups removed (g. $CH_3$/100 g. collagen) |
|---|---|---|---|
| | Untreated | | |
| *C* | 10·10 | 2·4 | — |
| *C* | 12·08 | 3·0 | — |
| *C* | 12·27 | 3·4 | — |
| *C* | 12·32 | 3·7 | — |
| *C* | 12·69 | 4·8 | — |
| *C* | 13·00 | 6·6 | — |
| *A* | 12·08 | 3·2 | — |
| | After methylation with dimethyl sulphate | | |
| $MC_4$ | 7·12 | 2·6 | 0·28 |
| $MC_4$ | 7·42 | 4·9 | 0·29 |
| $MC_4$ | 8·60 | 4·9 | 0·33 |
| $MC_4$ | 8·79 | 10·4 | 0·59 |
| $MC_4$ | 10·73 | 15·9 | 0·98 |
| $MC_4$ | 11·80 | 21·5 | 1·01 |
| $MA_2$ | 12·09 | 4·9 | — |
| $MA_3$ | 12·12 | 6·3 | — |
| $MA_4$ | 12·10 | 8·3 | — |
| *MDA* | 12·16 | 5·7 | — |
| | After methylation with methyl bromide | | |
| $MC_5$ | 8·76 | 1·1 | 0·32 |
| $MC_5$ | 9·28 | 1·1 | 0·27 |
| $MC_5$ | 10·20 | 1·1 | 0·33 |
| $MC_5$ | 11·61 | 1·6 | 0·20 |
| $MC_5$ | 12·01 | 1·5 | 0·34 |
| $MC_5$ | 12·24 | 2·1 | 0·33 |

At high pH values loss of methoxyl groups from collagen methylated with methyl sulphate is accompanied by the production of an appreciable amount of nitrogen in the solution (see Table 3). Collagen methylated with methyl bromide, on the other hand, gave less soluble nitrogen than the untreated collagen. The solutions smelt of methylamines and gave a positive test for dimethylamine (Dowden, 1938). This smell was also noted by Blackburn *et al.* (1941) during the methylation of wool.

It was considered possible that the N in solution was related to the methylation of one of the basic groups of the collagen and specific degradation of this group on treatment in alkaline solution. The arginine content of the collagen as determined by Vickery's method, however, was unchanged by methylation and treatment with alkali, suggesting that the soluble N was not derived from guanidino groups. (There is some evidence, however, that some methylation of guanidino groups does take place. Lindley & Phillips (1947) found no decrease in the arginine content of methylated wool as determined by Vickery's method, but tests on the flavaniate precipitate indicated that some change had occurred.) The solution gave only a slight positive reaction for protein, and the results as a whole suggest that the soluble nitrogen is derived from general hydrolysis of the collagen to polypeptides and amino-acids.

### *Titration curves*

*Deaminated collagen.* The titration curve of deaminated collagen ($DC_2$) is given in Fig. 2. Deamination has caused a shift in the isoelectric point from 5·5 to 4·5, a decrease in the acid-binding capacity, an increase in the base-binding capacity immediately on the alkaline side of the isoelectric point, and a slight increase in the total base bound at pH 12·5. The decrease in acid binding can be attributed mainly to loss of amino groups, but consideration of the free amino N of the original collagen (0·38 mmol./g.) shows that at least 0·07 mmol./g. of some other basic group has also been affected. The increase in base-binding from the isoelectric point to pH 7·0 corresponds to the decrease in acid-binding capacity, and may be attributed to titration of carboxyl groups which have reverted to the uncharged form on the removal of the basic groups. The increase in base-binding capacity at pH 12·5 indicates that about 0·18 mmol./g. of some group or groups not titrating in the original collagen is now binding base between pH 7 and 12·5.

*Methylated collagen.* The titration curves of collagens $MC_2$ and $MC_4$ (methylated with dimethyl sulphate) and $MC_5$ (methylated with methyl bromide) are shown in Fig. 2. In agreement with the hypothesis that methylation involves esterification of ionized carboxyl groups which are the hydrogen ion-fixing groups in the original protein, whilst the basic groups remain charged, the acid-binding capacities of the methylated collagen are decreased by an amount approximately corresponding to their methoxyl contents. The decrease in acid-binding capacity, however, is slightly less than the methoxyl content of the collagen, possibly owing to

loss of methyl groups by hydrolysis during contact with the solution. Also, since the isoelectric point of the methylated collagen is about one pH unit higher than that of the original collagen, some of the imidazole groups of histidine which were previously charged, and, therefore, titrated with alkali, will have lost their charge and now contribute to the acid-binding capacity.

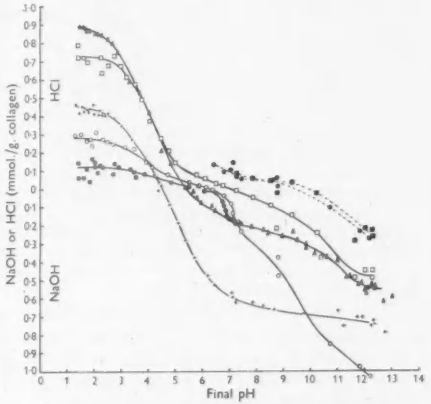


Fig. 2. Titration curves of collagen and modified collagens. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate, $CH_3$ content 1·04%; •—•, esterified with methyl sulphate, $CH_3$ content 1·04%; corrected for hydrolysis of $OCH_3$ groups; ⊗—⊗, esterified with methyl sulphate, $CH_3$ content 1·57%; ⊡—⊡, esterified with methyl bromide, $CH_3$ content 0·35%; ■—■, esterified with methyl bromide, corrected for $OCH_3$ hydrolysis; ⊕—⊕, deaminated and esterified with methyl sulphate, $CH_3$ content 1·29%.

The alkaline section of the curve is more difficult to interpret for, except over a comparatively small pH range, hydrolysis of methoxyl groups takes place with the consumption of alkali. The methoxyl content of the collagens, after contact with the solution, was determined and a correction applied for the alkali consumed; this is only approximate, but the corrected results suggest that appreciably less alkali is bound by the methylated than by the original collagen. In the case of collagen methylated with methyl bromide, the titration curve shows that less alkali is bound even before the correction is applied.

### *Swelling curves*

*Deaminated collagen.* The uptake of water by the deaminated collagen (see Fig. 3) differs from that of the original collagen in a manner broadly corresponding to the differences in the titration curves (Fig. 2), but at all pH values the water uptake is rather greater than would be expected from consideration of the changes in the reactive groups. This suggests that deamination has decreased the cohesion of the collagen so allowing it to take up more water.

*Methylated collagen.* In the pH range of stability of the methoxyl groups, i.e. 2–9, the water uptake of methylated collagen is constant, and, when the collagen is fully methylated, is approximately the same as the maximum water uptake of the untreated collagen at pH 2·0 (see Fig. 3). This high water uptake over such a wide pH range may be ascribed to the presence of anions in electrovalent association with the basic groups, and consequent setting up of a Donnan membrane equilibrium and a swelling pressure (Donnan, 1911, 1924; Bolam, 1932). In this respect, methylated collagen corresponds to collagen at the point of maximum combination with acid.

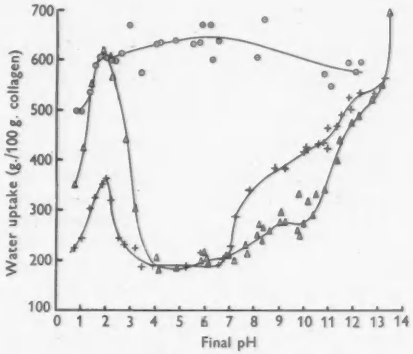


Fig. 3. The uptake of water by untreated △—△, deaminated +—+, and methylated collagen ⊙—⊙, at pH values from 0·5 to 13·5.

### *Combination of untreated and modified collagens with tannic acid, mimosa tannins, chromium and formaldehyde*

*Tannins.* The conditions of treatment were the same for all the modified collagens, and it is reasonable, therefore, to assume that any differences in the amount of tannin bound are primarily due to modification of the collagen.

Modification of the amino and carboxyl groups of the collagen affects the combination of both tannic acid (hydrolyzable tannin) and mimosa (condensed tannin) with collagen in a similar manner (Figs. 4–6). Deamination decreased the amount of tannin bound at pH values below 4·0, but had little effect at higher pH values, and esterification of the carboxyl groups caused maximum combination of tannin to take place at much higher pH values, and to remain constant at this maximum over a wide pH range; this was especially marked with mimosa tannin (Fig. 6).

The addition of 0·5 M-NaCl decreased the amount of tannic acid bound by untreated and deaminated

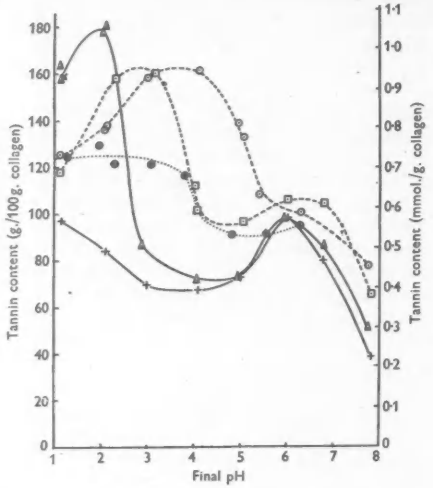


Fig. 4. Combination of tannic acid with collagen and modified collagen in the absence of added NaCl. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate; ⊡—⊡, esterified with methyl bromide; ⊕—⊕, deaminated and esterified with methyl sulphate.

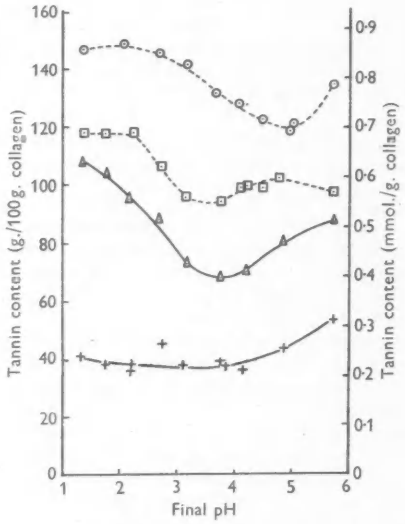


Fig. 5. Combination of tannic acid with collagen and modified collagen in the presence of 0·5 M-NaCl. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate; ⊡—⊡, esterified with methyl bromide.

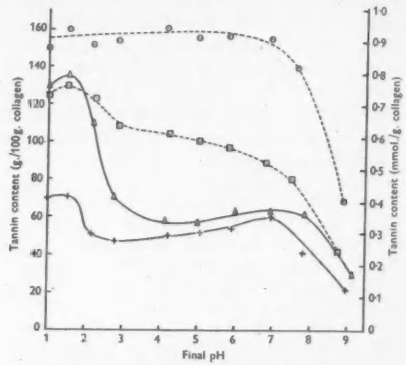


Fig. 6. Combination of mimosa tannins with collagen and modified collagen in the absence of added NaCl. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate; ⊡—⊡, esterified with methyl bromide.

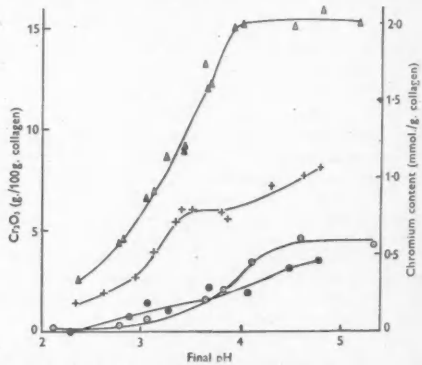


Fig. 7. Combination of chromium with collagen and modified collagen. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate; ⊕—⊕, deaminated and esterified with methyl sulphate.

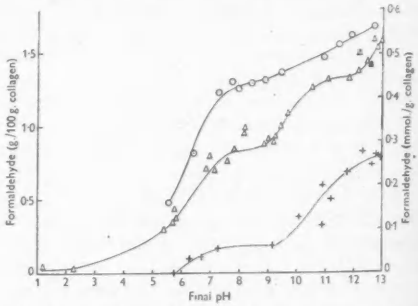


Fig. 8. Combination of formaldehyde with collagen and modified collagen. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate.

collagen, and by collagen methylated with methyl bromide, especially at low pH values. The tannic acid bound by the collagen methylated with methyl sulphate was only slightly decreased at pH values below 5·0, and at higher pH values was appreciably increased by the presence of NaCl.

The shrinkage temperatures of the collagen and modified collagen were increased from about 60 to between 66 and 84° by combination with tannic acid or mimosa tannin, maximum values in general being obtained when the pH of the tannin solution was between 3·0 and 4·0. The increase in shrinkage temperature was rather greater with mimosa tannin than with tannic acid, possibly because of the lower acidity of mimosa tannin (Hobbs, 1940). The deaminated and methylated collagens tended to have lower shrinkage temperatures than the original collagen treated under the same conditions, and variations of shrinkage temperature with the pH of the tannin solutions were less.

*Chromium.* The chromium bound by the original collagen increased sharply with increase in pH of the chromium sulphate solution up to 4·0, and then remained constant over the pH range studied (Fig. 7). Deamination decreased the combination of chromium over the whole pH range and reduced the maximum amounts combined by approximately half. Methylation decreased combination to a much greater extent. Chromium hydroxide was precipitated on the surface of the methylated collagens at the higher pH values and was difficult to remove; this, coupled with the possibility that some groups are displaced by chromium during the treatment, makes it not unreasonable to assume that in the absence of carboxyl groups no chromium would be bound by collagen. Deamination of the methylated collagen caused only a further small decrease in the chromium bound. Although the amount of chromium bound by the deaminated collagen was less than that bound by the original collagen, the maximum shrinkage temperature obtained was the same. Treatment with chromium sulphate caused no change in the shrinkage temperature of methylated colagen.

*Formaldehyde.* At pH values below 2·0, negligible amounts of formaldehyde were bound by both the untreated and modified collagens: from pH 2 to 8 the amount bound increased, remained constant between pH 8 and 9, and then increased again at higher pH values (see Fig. 8). Deamination greatly reduced combination between pH 2 and 9. The amount of formaldehyde bound by the untreated collagen up to pH 9·0, and the decrease in combination following deamination, correspond to considerably less than the combination of one formaldehyde molecule by each amino group; similarly, the amount bound from pH 9·0 to 13·0, the upper limit of pH covered, only corresponds to the combination of one molecule of formaldehyde by each of half the total number of guanidino groups.

With untreated and methylated collagen the combination of as little as 0·4 g. formaldehyde/100 g. raised the shrinkage temperature to its maximum value (78–80°), whereas the combination of 0·8 g. formaldehyde/100 g. left the shrinkage temparature of the deaminated collagen unchanged.

## DISCUSSION

*Deamination.* Under the experimental conditions employed, treatment with nitrous acid removed almost all the amino groups from collagen and decreased the arginine content by about 20 %. The course of the reaction with the amino groups is well known, but little attention appears to have been given to the reaction with the guanidino groups. Bancroft & Belden (1931) and Bancroft & Ridgway (1931) have shown that guanidine reacts with nitrous acid to give cyanamide and ammonia, and it is possible that a similar reaction takes place with the guanidino groups in proteins, namely,

$$—CH_2.NH.C\begin{matrix}NH_2\\ \diagdown\\ \diagup\\ NH\end{matrix} + HONO \rightarrow —CH_2.NH.CN + NH_3.$$

In agreement with this hypothesis, Kanagy & Harris (1935) have shown that ammonia is formed when arginine is treated with nitrous acid; this ammonia will react with nitrous acid to give the excess nitrogen observed in the Van Slyke determination of amino N (Plimmer, 1924; Hunter, 1929; Kanagy & Harris, 1935; Lieben & Loo, 1942; Van Slyke, Hiller & Dillon, 1942). This hypothesis would also explain the higher amide N of the deaminated collagen compared with the original collagen since, under the conditions of the determination, the —NH.CN group would probably yield ammonia. This increase in amide N is of the same order as the decrease in the arginine content of the collagen.

The reaction postulated above is also consistent with those changes in the titration curve which cannot be attributed to loss of amino N. Loss of the strongly basic guanidino groups accounts for the decrease in acid-binding capacity; carboxyl groups equivalent to the guanidino groups lost will revert from the zwitterion state (—$COO^-$) to the uncharged state, and will titrate between the isoelectric point and pH 7·0, and the —NH.CN groups, being feebly acidic, will titrate between pH 7 and 12, thus accounting for the increased base-binding capacities in these ranges. Since guanidino groups do not titrate with base up to pH 12·5 (Bowes & Kenten, 1948*a*), the total base-binding capacity should be increased by an amount equal to twice the —NH.CN groups formed, i.e. base bound by the additional

un-ionized carboxyl groups plus that bound by —NH.CN groups. On this basis, and assuming that 0·05 mmol./g. amino groups remain (see Table 1), the curve indicates that about 0·10 mmol./g. guanidino groups are converted to cyanide groups. (Decrease in acid binding in excess of that due to amino groups, 0·12 mmol./g.; base bound between 7 and 12, 0·10 mmol./g., and total increase in base-binding capacity, 0·20 mmol./g.) This figure is in good agreement with decrease in arginine content found by analysis, 0·09 mmol./g.

*Esterification.* Like the carboxyl groups of amino-acids, those of wool, silk, gelatin, and collagen can be esterified with methyl sulphate and methyl bromide (Blackburn *et al.* 1941; Blackburn & Phillips, 1944).

The evidence obtained in the present investigation confirms this view with respect to collagen. The reaction would appear to necessitate the presence of basic groups equivalent to carboxyl groups; it was found, however, that deamination did not decrease the extent of methylation. Blackburn & Phillips (1944) considered that the lysine content of wool is so low that its removal does not materially affect the number of basic groups. This explanation is not applicable to collagen since amino groups form about one third of the total basic groups, and a more likely explanation is that as ionized carboxyl groups are esterified, further carboxyl groups ionize under the influence of the guanidino groups, so that eventually all carboxyl groups are esterified.

Consideration of the values for the free carboxyl groups in the untreated collagens, as indicated by analysis and by their titration curves (Bowes & Kenten, 1948*b*), shows that all the methoxyl groups introduced can be accounted for on the basis of esterification of carboxyl groups. In three experiments, the number of methoxyl groups was slightly in excess of the number of free carboxyl groups but this excess was within the experimental error. Blackburn *et al.* (1941) and Blackburn & Phillips (1944) found that the methoxyl groups introduced by methyl sulphate exceeded the number of free carboxyl groups believed to be present in wool, silk, gelatin, and collagen, and suggested that methylation also occurred at certain 'activated' peptide links. It is now known (Bowes & Kenten, 1948*a*) that the values taken for the number of carboxyl groups in gelatin and collagen were low, and the present work shows that with collagen there is no necessity to postulate any form of *O*-methylation other than that of carboxyl groups. It is possible that future determinations of the dicarboxylic acids in wool and silk will obviate the necessity of assuming that these proteins undergo peptide methylation.

The low amino-nitrogen values of the methylated collagens suggest that some *N*-methylation has occurred, especially with methyl bromide. The lower base-binding capacity of the methylated collagens as compared with that of untreated collagen may also be due to *N*-methylation, for although the introduction of a methyl group will not eliminate the basic characteristics of the amino group, it will, by analogy with the methylamines, increase the pK of these groups and hence cause a shift in the titration curve to higher pH values. It is possible that the titration curves of the methylated collagen would show the same maximum base-binding capacity as the original collagen if carried to higher pH values. It is also possible that the reduction in base-binding capacity of the collagen methylated with methyl sulphate is related to the presence of nitrogen in the solutions after contact with the collagen; if this nitrogen were present as a base (for instance, as methylamine) it would titrate and so reduce the apparent fixation of base by the collagen.

*Combination of collagen and modified collagens with tannins, chromium and formaldehyde.* Although tannic acid, in contrast to the tannins of mimosa, contains acidic groups (Sunthankar & Jatker, 1938; Abichandani & Jatker, 1938; Cheshire, Brown & Holmes, 1941) which might be expected to form salts with the basic groups of the collagen, the mode of combination with collagen appears to be the same with both materials. The results as a whole are consistent with the supposition that combination of tannin is related to the positive charge carried by the protein. With the untreated collagen, combination is greatest between pH 1·5 and 2·0 when the collagen carries its maximum net positive charge, decreases as the pH increases and the net positive charge decreases, and eventually is reduced to negligible proportions at pH 8·0–9·0 when the basic groups begin to lose their positive charge and the protein carries a net negative charge. Deamination which decreases the positive charge on the collagen, decreases combination of tannin, and esterification, which causes the collagen to carry its maximum positive charge over the whole pH stability range of the methoxyl groups, causes combination of tannin also to be at a maximum over this range. Experiments indicate that the molecular weight of tannic acid and mimosa tannin is 1700 (Brintzinger & Brintzinger, 1931; Humphreys & Douglas, 1937), and the equivalent weight of tannic acid is of the same order (Cheshire *et al.* 1941). Using these figures, an interesting comparison can be made between the amounts of tannin bound and the acid-binding capacity of the collagens (see Table 4). The molecular amounts of tannin combined with the untreated and deaminated collagens between pH 1·5 and 2·0 are of the same order as their acid-binding capacities; deamination halves the acid-binding capacity and also halves the amount of tannic acid and mimosa tannin bound. This suggests that one molecule of tannin is associated with each basic

group. Although the amounts of tannin bound are dependent on the concentration of tannin in the solution, the time of treatment and method of washing, it would seem reasonable to assume that there is some significance in these stoicheiometric relationships.

Table 4. *Acid and tannin bound by collagen and deaminated collagen*

(Results in mmol./g.)

| Collagen | Acid-binding capacity | Tannin bound between pH 1·5 and 2·0 | |
|---|---|---|---|
| | | Tannic acid | Mimosa tannin |
| Untreated collagen *A* | 0·88 | 1·0 | 0·80 |
| Deaminated collagen $DA_1$ | 0·44 | 0·50–0·55 | 0·41 |

The present results emphasize the essential part played by the carboxyl groups in the binding of chromium, and strengthen the current view that the fixation of metals involves complex formation with the carboxyl groups of the protein in a manner analogous to the complex formation which occurs with the carboxyl groups of organic acids (for review of literature see Bowes, 1948). The amino groups also appear to be involved since deamination decreased the amounts bound by the untreated collagen, though not by the methylated collagen. These findings are consistent with the hypothesis that combination of chromium involves co-ordination of both carboxyl and amino (or other basic groups) with the same chromium complex. A similar hypothesis has been put forward to account for the fixation of calcium by proteins (Greenberg, 1944), and for the high thermal stability of chrome-tanned leather (Küntzel & Riess, 1936).

Although the validity of stoicheiometric relationships between the amounts of chromium bound and the reactive groups of the collagen may, in view of the complexity of the system, be open to question, it is of interest to note that the amount of chromium bound between pH 4 and 5 approximately corresponds to the fixation of two chromium atoms by each carboxyl group, and the decrease in combination caused by treatment with nitrous acid corresponds approximately to two chromium atoms for each basic group lost. It is probable that the chromium aggregates in the tanning solutions used contained, on the average, two chromium atoms (Bowes, 1948).

The evidence suggests that, in collagen, the amino and guanidino groups are the main centres involved in the binding of formaldehyde, and there is no evidence that the amide groups are concerned under the conditions employed. The importance of the amino groups raising the shrinkage temperature (Gustavson, 1943) is confirmed. Free amino groups also play an essential part in the hardening of casein with formaldehyde (Nitschmann & Hadorn, 1944; Nitschmann & Lauener, 1946), and it is probable that a similar mechanism is involved, namely, the formation of cross links between adjacent polypeptide chains. Nitschmann & Hadorn (1944) and Nitschmann & Lauener (1946) discuss the various ways in which such cross links may be formed and consider that a cross link between $\epsilon$-$NH_2$ groups and —NH— groups of the peptide link is the most probable. (For further discussion on this point see Fraenkel-Conrat, Cooper & Olcott, 1945; French & Edsall, 1945; Bowes, 1948.)

## SUMMARY

1. Collagen has been deaminated with nitrous acid and esterified with methyl sulphate and methyl bromide, and the effect of these treatments on the reactivity of collagen towards acids, bases, tannins, chromium and formaldehyde has been determined. Modification of the guanidino groups by treatment with hypochlorite (Sakaguchi (1925) reaction) was only partially successful; only 40–50% of the arginine was destroyed and there was extensive general breakdown of the collagen.

2. Treatment with nitrous acid removed almost all the amino groups from collagen and decreased the arginine content by approximately 20%. Evidence deduced from analysis and titration curves suggests that the guanidino groups are converted into cyanamide groups.

3. All the *O*-methyl groups introduced into collagen by methyl sulphate and methyl bromide can be accounted for on the basis of esterification of carboxyl groups. There is an indication that some *N*-methylation occurs, especially with methyl bromide.

4. From a study of the combination of tannins, chromium and formaldehyde with untreated, deaminated and methylated collagen, it is suggested that combination of tannins is related to the positive charge carried by the collagen, combination of chromium involves co-ordination of both amino and carboxyl groups of the collagen with the same chromium complex, and combination with formaldehyde occurs mainly with the amino and guanidino groups. Increase in thermal stability results only from combination of formaldehyde with amino groups.

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# The Behaviour of *Lactobacillus arabinosus* towards Nicotinic Acid and its Derivatives

By H. McILWAIN, D. A. STANLEY and D. E. HUGHES
*Unit for Cell Metabolism (Medical Research Council), Department of Biochemistry, University of Sheffield*

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These investigations originated in studying the basis of a method for microbiological assay of nicotinic acid (McIlwain & Stanley, 1948). The method was that in which acid produced from glucose by *Lactobacillus arabinosus* 17–5 is titrated with alkali after a period of incubation of about 3 days. The assay depends on the empirical observation that the quantity of titratable acid produced during incubation is dependent on the quantity of added nicotinic acid. It was found (McIlwain, 1948) that this relationship existed because of changes which took place in nicotinic acid during the assay; the compound was first assimilated to, and later lost from, the cells. Its loss was found to be conditioned by its functioning as a catalyst, and was such that nicotinic acid exhibited a defined catalytic capacity, measured by the ratio: titratable acid formed/nicotinic acid lost (in mol./mol.). Values of the catalytic capacity (about $5 \times 10^5$) were relatively independent of the intensity with which nicotinic acid derivatives were functioning as catalysts, which was expressed as a catalytic activity (in mol. titratable acid/hr./mol. nicotinic acid; values ranged from 5 to $15 \times 10^3$ hr.$^{-1}$).

Such observations suggested the need for more detailed investigations of the form in which nicotinic acid derivatives existed in the bacterial cells. We have now examined the nicotinic acid of cells of *Lb. arabinosus*, and found it to be largely in the form of cozymase. *Lb. arabinosus*, in marked distinction from a number of other organisms, reacted only slowly with added cozymase; this, and our interest in the practical problems presented by microbiological assay, directed our attention to metabolic changes occurring in the cozymase of the bacterial cells, and these are described below.

## EXPERIMENTAL

The assays for nicotinic acid, the preparation of growth media and of materials for assay, and the handling of non-proliferating suspensions of *Lb. arabinosus* were carried out as described by McIlwain (1948). Determination of V-factor and of cozymase, and the extraction of these substances from bacteria were made by the methods used by McIlwain & Hughes (1948).

## RESULTS

### *Assimilation of nicotinic acid by* Lactobacillus arabinosus

1. *Cell content.* Cells of *Lb. arabinosus* from cultures whose growth has not been limited by nicotinic acid may, nevertheless, contain varying quantities of this substance. This is shown in Table 1. Nicotinic acid becomes a limiting factor in growth of the organism at concentrations of about $2 \times 10^{-7}$ M and the range used in assay is below $1{\cdot}5 \times 10^{-7}$ M. The values of Table 1 illustrate also the previous findings (McIlwain, 1948) that the nicotinic acid content of the cells of a given culture falls with time. To approach saturation of the cells with the acid at 26 hr. in the relatively early part of a culture's growth, a concentration of *c.* $5 \times 10^{-7}$ M-nicotinic acid is seen to be required. The cells will thus be unsaturated in this sense during most of their period of incubation in all the concentrations used in assay.

Under the conditions most favourable for assimilation, about 80 % of the nicotinic acid added to a culture has been recovered from the cells after 26 hr. growth. As the cells inactivate nicotinic acid during this period, this result gives a minimum estimate only of the completeness with which they can assimilate the acid. The actual concentration of nicotinic acid derivatives in the cells after assimilation can be calculated from the data of Table 1 to be about 2 to $10 \times 10^{-4}$ M. In making this calculation the wet weight of the cells was taken as five times their dry weight; this has been found to be a typical ratio. The values recorded in Table 1 for the yield of bacterial cells from unit volume of culture show that the assay medium, though carefully chosen for maximum response to limited nicotinic acid, does not give high yields of bacteria.

2. *Nature of the assimilated nicotinic acid.* Most of the nicotinic acid of the cells existed as derivatives, especially in cells containing limiting quantities of the substance. Table 2 A shows that some 90 % of the acid, determined in cells grown from a concentration of the acid within assay range, exists as

Table 1. *Nicotinic acid content of cells of* Lactobacillus arabinosus *after growth of varying types*

| Nicotinic acid of growth medium (M × $10^{-7}$) | Period of incubation (hr.) | Yield of cells (mg. dry wt./ 100 ml. culture) | Nicotinic acid content of cells | | |
|---|---|---|---|---|---|
| | | | (mμmol./cells of 100 ml. medium) | (mμmol./mg. dry wt.) | In fresh cells* (M × $10^{-4}$) |
| 1·2 | 30 | 7·0 | 6·2 | 0·88 | 1·8 |
| 2·4 | 26 | 10·4 | 13·9 | 1·33 | 2·7 |
| 4·9 | 26 | 9·5 | 37 | 4·04 | 8·1 |
| 4·9 | 42 | 10·7 | 33 | 3·06 | 7·1 |
| 4·9 | 48 | 12·0 | 28 | 2·34 | 4·7 |
| 12·2 | 26 | 9·8 | 53 | 5·4 | 10·8 |

* Calculated, assuming a water content of 80%.

Organisms were grown in quantities of 200–500 ml. of the assay medium (cf. McIlwain & Stanley, 1948) with nicotinic acid added in the quantities indicated.

Table 2. *Nicotinic acid derivatives in cells of* Lactobacillus arabinosus, *and in material liberated from cells to solution*

| Nicotinic acid in growth medium (mμmol./100 ml.) | Period of incubation in growth (hr.) | Yield of cells (mg. dry wt./ 100 ml. culture) | Later treatment | Content (mμmol./cells of 100 ml. culture) | | |
|---|---|---|---|---|---|---|
| | | | | Nicotinic acid | V-factor | Cozymase |
| | | | A. Material in cells | | | |
| 12·2 | 30 | 13·0 | Washed | 6·9 | — | 6·5 |
| 12·2 | 30 | 13·0 | Washed, and in saline mixture* at 37°, 6 days | 2·7 | — | 2·4 |
| 45 | 28 | 10·9 | Washed | 19 | 14 | — |
| 122 | 48 | 12·8 | Washed | 43 | 27 | — |
| | | | B. Material liberated on exposure of cells in salt mixture† | | | |
| 45 | 28 | 10·9 | Cells washed and | 6·1 | 4·2 | — |
| 112 | 48 | 12·8 | exposed† | 16·4 | 8·4 | — |

* The inorganic salts of the medium of Barton-Wright (1946) at pH 6·8 and in the concentrations of the final assay medium.

† In the inorganic salts (organisms from 100 ml. of culture in 50 ml. salt solution) for 72 hr. at 37°, aerobically.

cozymase. The amount determinable as V-factor (probably equivalent to nicotinamide ribosides: for discussion see McIlwain, 1947) was also high.

3. *Relative stability of cozymase added to* Lb. arabinosus. McIlwain & Hughes (1948) found that many bacterial species rapidly inactivate added cozymase; even at low concentrations of cozymase (*c.* $10^{-5}$ M) loss may occur at the rate of 10 mμmol./mg. dry wt./hr., and with higher concentrations may rise to over 500 mμmol./mg./hr. Such a coefficient means that the organism is inactivating its own weight of cozymase in about 3 hr. *Lb. arabinosus* was, however, relatively inert towards cozymase. Under the conditions of its use in assay, i.e. with cells of between 1 and 3 days growth, and at pH values between 5 and 7, any reaction with $10^{-5}$ or $10^{-4}$ M-cozymase was of $< 1$ mμmol./mg. dry wt. of cells/hr. In attempts to observe a more rapid reaction, experiments were also carried out at pH 4·5 and 7·6, in the presence and absence of phosphates and of glucose, and with some suspensions initially rich and others initially poor in nicotinic acid derivatives. Again, any reaction was of $< 1$ mμmol./mg./hr. It was evident that *Lb. arabinosus* did not inactivate cozymase at the rapid rate observed in several other bacteria. Nevertheless, slow inactivation has been observed (Table 1), and found to be related to the assay (McIlwain, 1948); this process has, therefore, been studied further.

*Change in nicotinic acid in suspensions of* Lactobacillus arabinosus

An attempt was made to reproduce the changes of Table 1 under conditions better defined than those of a growing culture. Table 3 shows that the cellular nicotinic acid of *Lb. arabinosus* can also be very stable. Cells were examined which had been grown in media relatively rich and relatively poor in nicotinic acid; the latter conditions (12 mμmol. nicotinic acid/100 ml.) are within the range used in assay. In all cases the cells could be incubated at 37° in salt mixtures at pH 6·5–7 for 6 days without any loss in the total nicotinic acid of the suspension. A small increase, of some 10% of the initial value, was frequently found.

These experiments showed that the loss in nicotinic acid observed during the assay was not brought about by the bacteria when they were incubated in the presence of the majority of the constituents of the assay medium. The components omitted from the suspending fluids during the

experiments quoted were glucose, the casein hydrolysate providing amino-acids to the medium, and in one case sodium acetate. Other results in Table 3 showed sodium acetate to have only a small effect in the change in nicotinic acid; but addition of glucose caused a major change. In each case examined, addition of glucose led to a decrease in nicotinic acid content.

*The course of changes in nicotinic acid derivatives in suspensions of* Lactobacillus arabinosus

1. *In absence of glucose.* Although the total nicotinic acid of cell suspensions changed little under these conditions, marked changes occurred in its distribution. Fig. 1 shows this in the case of a suspension of cells which were initially rich in the

Table 3. *Changes in the nicotinic acid derivatives of suspensions of cells of* Lactobacillus arabinosus

| Batch of organisms; conditions of growth | Initial content of nicotinic acid (mμmol./mg. dry wt.) | Conditions of reaction | Change in nicotinic acid during 6 days* (mμmol./mg. dry wt.) |
|---|---|---|---|
| 1. 26 hr., 122 mμmol. nicotinic acid/100 ml. | 5·4 | Salts of medium,† pH 6·8 | +0·70 |
| | | As above, with glucose (0·1 M) | −1·80 |
| 2. 30 hr., 12 mμmol. nicotinic acid/100 ml. | 0·88 | Salts of medium, pH 6·5 | +0·10 |
| | | As above, with glucose (0·1 M) | −0·25 |
| 3. 26 hr., 24 mμmol. nicotinic acid/100 ml. | 0·72 | Inorganic salts of medium,‡ pH 7 | +0·08 |
| | | As above, with glucose (0·1 M) | −0·14 |

* The change quoted is in the whole reaction mixture; the kinetics of the change and the partition of nicotinic acid between cells and solution are discussed later.
† NaCl, Na acetate, and inorganic salts A and B of Barton-Wright (1946), at the concentrations used in assay.
‡ As in footnote †, but lacking Na acetate.

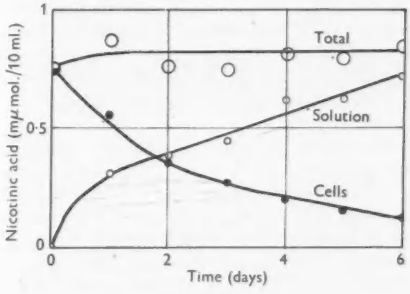


Fig. 1.

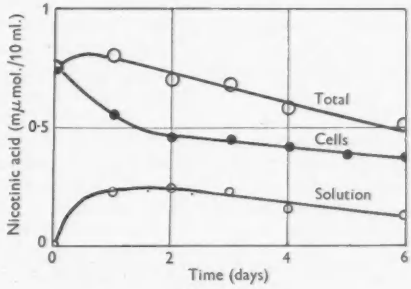


Fig. 2.

Figs. 1 and 2. Change in distribution of nicotinic acid in suspensions of cells of *Lb. arabinosus*, grown with 122 mμmol. of the acid/100 ml. Cells were harvested after 26 hr. growth, and washed with, and suspended in, the inorganic salts of the growth medium without glucose (Fig. 1) and with 0·1 M-glucose (Fig. 2).

Although the effect of glucose was marked, the absolute magnitude of the change which it caused was small. It led to loss of about 30% of the cells' nicotinic acid in 6 days, but this involved an average rate of loss over that period of only 2–7 μμmol./mg. dry wt. of cells/hr. The change in a batch of cells grown from 12 mμmol. nicotinic acid/100 ml., and thus within the assay range, was about −2·5 μμmol./mg./hr., and thus much slower than that estimated as occurring during assay. This led us to investigate the course of the change, the distribution of nicotinic acid between cells and solution, and the effect of additional substances on the changes.

acid. The quantity in the cells fell throughout the experiment, but at a decreasing rate; and the acid which was lost from the cells appeared in the solution. Results similar to those of Fig. 1 have been obtained also with cells which were initially poorer in nicotinic acid (containing 30 or 15 mμmol./100 ml., the latter value being the upper limit of the assay range); but in such cases the proportion of nicotinic acid leaving the cells was smaller than with those initially rich in the acid.

The nicotinic acid-containing material liberated from the cells consisted at least in part of derivatives of the acid. The solutions yielded were too dilute to give significant values for cozymase in the apozy-

mase system, but their V-factor activity could be determined. The values obtained (Table 2B) showed two thirds or one half of the liberated material to possess such activity and presumably to be at least of the complexity of a riboside of nicotinamide.

2. *In presence of glucose.* Glucose led not only to the decrease in total nicotinic acid noted in the previous section, but in many cases it also caused a marked change in the distribution of nicotinic acid derivatives between cell and solution. Fig. 2 shows that, with organisms rich in nicotinic acid, glucose caused nicotinic acid which otherwise would have passed into solution to be retained in the cells. The quantity in solution increased only transitorily, and then fell in a fashion roughly parallel to that of the cells. This behaviour was less marked in organisms which initially contained less nicotinic acid, and with cells grown from 12 mμmol. of the acid/100 ml. no considerable change in distribution of the acid was brought about by glucose (Table 4), but the fall in total quantity remained marked.

Table 4. *Effect of glucose on distribution of nicotinic acid in suspensions of* Lactobacillus arabinosus

| Conditions of growth and reaction | Nicotinic acid (mμmol./10 ml. of suspension) Initially | After 6 days: Cells | After 6 days: Solution | Percentage of nicotinic acid present in cells after 6 days |
|---|---|---|---|---|
| As 1, Table 3; no glucose | 4·9 | 0·81 | 4·8 | 14 |
| As 1, Table 3; with glucose | 4·9 | 2·44 | 0·9 | 73 |
| As 2, Table 3; no glucose | 3·05 | 1·06 | 2·26 | 32 |
| As 2, Table 3; with glucose | 3·05 | 1·38 | 1·0 | 58 |
| As 3, Table 3; no glucose | 0·88 | 0·49 | 0·45 | 52 |
| As 3, Table 3; with glucose | 0·88 | 0·29 | 0·26 | 53 |

### *Acid formation from glucose by suspensions of* Lactobacillus arabinosus

1. *Fall with time.* The changes in cellular nicotinic acid are relevant to the assay because this depends on acid formation by the cells, and this again depends on the presence of nicotinic acid derivatives. It has already been shown (McIlwain, 1948) that freshly harvested cells of *Lb. arabinosus* are capable of fermenting glucose at rates almost equal to the most rapid reached during growth in nutritionally rich media. Organisms from nicotinic acid-deficient cultures were found to produce acid at a lower rate than those with excess of the acid, and the deficiency could be made good by addition of nicotinic acid or cozymase. In these experiments acid formation was followed for only an hour or two after harvesting.

The course of acid formation from suspensions of *Lb. arabinosus* was now followed over periods comparable to those of the assay. Fig. 3 shows that observed in an experiment lasting 6 days. Organisms had been grown from a quantity of nicotinic acid (1·22 mμmol./10 ml.) within the assay range and were reaped at 30 hr. when their rate of acid formation was high. Their initial rate in the salts of the assay medium, with glucose, was > 10 μmol./10 ml./hr., and this fell to about 1 μmol./10 ml./hr. on the sixth day. The rate fell rapidly during the first 2 or 3 days and then more slowly. Changes in the rate have, therefore, been examined in relation to changes in cellular nicotinic acid.

2. *Catalytic activity of nicotinic acid in cell suspensions.* Relevant results are presented in Table 5. Here the rate of acid formation in the suspensions is expressed as a multiple of the quantity of nicotinic acid in the cells. These values (catalytic activities: in mol. titratable acid/mol. nicotinic acid/hr.) are seen to be relatively constant but to fall during the course of exposure of the cells. The cells are thus changing during exposure so that nicotinic acid becomes progressively less effective as a catalyst. It is also initially much less effective as a catalyst during the present experiments than in assay; the catalytic activities of Table 5 are only about one tenth of those found (McIlwain, 1948) at comparable pH values during assay.

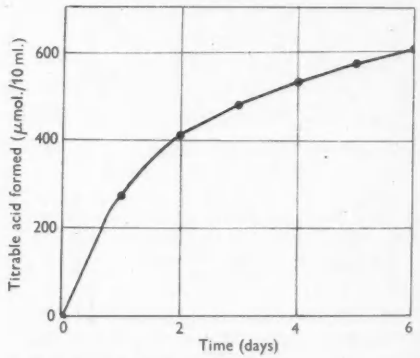


Fig. 3. Course of formation of titratable acid in suspensions of *Lb. arabinosus*, grown in the same way as the organisms of Figs. 1 and 2 but for 30 hr., and suspended in the medium described for Fig. 2.

The higher catalytic activity during assay did not appear to be due to cell proliferation, as the earlier experiments included ones of short duration with harvested and resuspended cells. The difference was found to be due to the organic constituents of the suspending fluid. Reconstituting the assay medium by adding to the suspending fluid of Table 5 casein hydrolysate, ammonium salts, and a mixture of vitamin-like substances restored the catalytic activity to 14,000 mol./mol./hr. at pH 6, or 8000 at pH 5·2. In this effect the vitamin-like substances played little part (Table 5).

their cells. The assimilated material has been found to be present largely as cozymase, which is much more stable when added to suspensions of *Lb. arabinosus* than it is in suspensions of many other organisms. Loss of cozymase from buffered suspensions of *Lb. arabinosus* was extremely slow, both with respect to cozymase which was added as such, and also to that which was synthesized by the cells themselves from nicotinic acid. Cellular cozymase became markedly less stable in glycolyzing organisms although here also the rate of its loss was by most standards very low, being about 10 $\mu\mu$mol./mg. dry

Table 5. *Catalytic activity and catalytic capacity of nicotinic acid in acid formation by suspensions of* Lactobacillus arabinosus

(Cells were grown with 1·2 $\mu$mol. nicotinic acid/10 ml., reaped after 30 hr. incubation, washed twice aseptically with, and suspended in, the inorganic salts and buffers of the assay medium together with glucose but excluding the ammonium salts. Initial pH of medium 6·5; concentration of cells 2·95 mg./10 ml.)

| Age of suspension (days) | pH | Nicotinic acid content of cells (m$\mu$mol./10 ml. suspension) | Rate of formation of titratable acid ($\mu$mol./10 ml. suspension/hr.) | Catalytic activity (mol. titratable acid/ mol. nicotinic acid/hr.) | Catalytic capacity (mol. titratable acid formed/mol. nicotinic acid lost) ($\times 10^5$) |
|---|---|---|---|---|---|
| 1 | 5·7 | 2·44 | 9·1 | 3700 | 3·5 |
| 2 | 5·4 | 2·04 | 4·6 | 2300 | 2·1 |
| 3 | 5·3 | 1·70 | 2·62 | 1500 | 2·3 |
| 4 | 5·25 | 1·48 | 2·04 | 1400 | 2·0 |
| 5 | 5·2 | 1·28 | 1·52 | 1200 | 2·1 |
| 6 | 5·2 | 1·14 | 1·08 | 1000 | |

A parallel experiment was carried out with similarly grown organisms in a suspending fluid of the same initial pH but with in addition the aneurin, riboflavin, pyridoxin, pantothenate, *p*-aminobenzoic acid and biotin of the assay medium. Catalytic activities of 2000–1200 were observed, and catalytic capacities of $2{\cdot}5$–$3{\cdot}5 \times 10^5$ (mean, $2{\cdot}6 \times 10^5$).

3. *Catalytic capacity of nicotinic acid in cell suspensions.* In Table 5 are given also the ratios between the mol. titrable acid formed and the mol. nicotinic acid lost. These values (the catalytic capacities of nicotinic acid) were relatively steady during the experiments and were not far removed from the values observed in assay. Thus, different suspensions gave $2{\cdot}5$–$3{\cdot}5$ and $2{\cdot}0$–$3{\cdot}5 \times 10^5$, and in assay the capacity was about $5 \times 10^5$. It has been observed above that in the present experiments, as in assay, loss of nicotinic acid was associated with the presence of glucose. The values now quoted show that the quantitative relations between the metabolism of glucose and of nicotinic acid are also similar during the assay in non-proliferating suspensions, although the absolute rates of metabolism of each fell in the suspensions to only a fraction of their value in assay.

## DISCUSSION

The present studies have given further information on the behaviour of nicotinic acid in *Lactobacillus arabinosus*, and this behaviour may now be assessed in relation to the use of the organism in assay. The organisms, in growth, assimilated nicotinic acid from very dilute solutions and concentrated it in wt./hr. It was, therefore, very interesting to observe that this rate bore a relation to the rate of glycolysis similar to that previously observed in more active cells during assay. In both cases the catalytic capacity of nicotinic acid, or the ratio between the rates of change in titratable acid in nicotinic acid, was between 2 and $6 \times 10^5$. The possible significance of this has been discussed previously (McIlwain, 1948).

These values give another instance of relative stability in the catalytic capacity of nicotinic acid during marked changes in its catalytic activity (i.e. the rate of reaction which unit quantity of it catalyzes, in this case glycolysis, expressed in $\mu$mol./hr./$\mu$mol. nicotinic acid). The catalytic activity was markedly affected by the suspending fluid. This is understandable, as glycolysis involves many reactions, and the slowing of any one of these during the 6 days of an experiment could be reflected in the overall rate of reaction. The relative constancy of the catalytic capacity implies that, when glycolysis has fallen in rate, loss of nicotinic acid has also been correspondingly slower.

Data already available from various assay methods for nicotinic acid give further indications of the relative constancy of the catalytic capacity of the acid. As emphasized previously (McIlwain, 1948), the full catalytic capacity of nicotinic acid is

not used during an ordinary assay. In the instance investigated, normal assay practice, which allows reaction to continue until acid formation becomes slow, employs about half the full catalytic capacity of the added nicotinic acid. The standard dosage/response curve of assay, when expressed in mol. titratable acid/mol. nicotinic acid, thus gives a partial catalytic capacity of nicotinic acid. Values of this type have been collected in Table 6. This shows partial catalytic capacities from our own observations with *Lb. arabinosus* to range from $2{\cdot}6$ to $4{\cdot}8 \times 10^5$; those of other observers with this organism, from $1{\cdot}7$ to $2{\cdot}6$, and with *Leuconostoc mesenteroides*, from $4{\cdot}9$ to $6{\cdot}8 \times 10^5$. Fundamental importance is not attributed to the partial catalytic capacities which are expressed immediately by these values, but the latter are considered to indicate that the catalytic capacities themselves are also likely to be of the same order of magnitude under the various conditions chosen by the different observers whose results have been given in Table 6. This increases the significance of the catalytic capacity.

Table 6. *Partial catalytic capacities of nicotinic acid during assays*

| Source of data | Conditions of assay: Organism | Conditions of assay: Temp. of incubation | Partial catalytic capacity $\times 10^{5}$* |
|---|---|---|---|
| 12 assays in conjunction with present paper, and that of McIlwain (1948) | *Lactobacillus arabinosus* | 37 | 2·6–4·8; mean, 3·4; standard deviation, 0·65 |
| Barton-Wright (1946) | ,, ,, | 30 or 37 | 2·6 |
| Snell & Wright (1941) | ,, ,, | 30 | 2·2 |
| Roberts & Snell (1946) | ,, ,, | 37 | 1·7 |
| Krehl, Strong & Elvehjem (1943) | *Leuconostoc mesenteroides* | 30 | 4·9 |
| Johnson (1945) | ,, ,, | 30 | 6·75 |

* Molar ratio: titratable acid formed/nicotinic acid added, during the approximately linear portion of the dosage/response curve. Different media of growth were employed by the different investigators; titratable acid was in all cases determined after 3 days' growth.

## SUMMARY

1. *Lactobacillus arabinosus* 17–5, which is used in a microbiological assay of nicotinic acid, assimilated this acid from $10^{-8}$ or $10^{-7}$ M solutions during growth. Its cell content of the substance was then $0{\cdot}7$–$5$ m$\mu$mol./mg. dry wt. of cells, according to conditions of growth.

2. The assimilated substance existed in the cells largely as cozymase. Cozymase in *Lb. arabinosus*, or added to suspensions of this organism, was much more stable than in the presence of many other bacteria.

3. A slow reaction (of 2–20 $\mu\mu$mol./mg./hr.), leading to inactivation of cozymase and of its nicotinic acid moiety did, however, occur in *Lb. arabinosus* during glycolysis. Glycolysis altered also the distribution of nicotinic acid between saline solutions and the cells of the organism, leading to greater retention of the acid in the cells.

4. Rates of glycolysis and of loss in nicotinic acid varied in parallel in suspensions of *Lb. arabinosus*, as they had previously been found to do in assay. The ratio between the two rates (the catalytic capacity of nicotinic acid) had approximately the same value, of $3$–$6 \times 10^5$, in the two cases.

5. On the other hand, the activity of nicotinic acid as a catalyst was much lower in non-proliferating suspensions in simple media, than in assay. Catalytic activity and partial catalytic capacity have been used as measures of the behaviour of the organism during assays of the present type.

We are greatly indebted to Miss E. Ellis for assistance during these investigations.

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# Distribution of Glutamine and Glutamic Acid in Animal Tissues

By H. A. KREBS, L. V. EGGLESTON and R. HEMS
*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Sheffield*

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It is generally accepted that glutamine and glutamic acid, apart from serving as structural units in proteins and peptides, play a special role in the metabolism of animals, plants and micro-organisms. A few specific functions have already come to light (see the reviews of Archibald, 1945, 1947), but it is probably correct to say that the chief functions are still unknown. It was thought that a survey of the occurrence of glutamine and glutamic acid in biological material might assist in elucidating the part played by the two substances in metabolism, and the two substances were, therefore, determined in a number of animal tissues. Surveys of the distribution of glutamine have been made by previous workers (Ferdman, Frenkel & Silakova, 1942; Hamilton, 1945), but data on the glutamic acid content of tissues are scanty because, until recently, no specific and convenient methods applicable to small quantities of material were available.

## EXPERIMENTAL

The procedure described recently (Krebs, 1948) was used. All tissues except blood were frozen in liquid air as soon as possible, usually within 2 or 3 min. after death. A delay of about 5 min. was unavoidable in the case of sheep brain and of about 20–40 min. in the case of foetal material. A quantity of about 5 g., in some cases less, was weighed out in the frozen state, and crushed in a mortar with 2 vol. of 0·5 N-HCl and washed sand. Blood was collected from fasting hospital patients, mixed with heparin and immediately centrifuged. The plasma (10 ml.) was shaken *in vacuo* after addition of 0·25 ml. N-HCl to remove bicarbonate and $CO_2$. Two Warburg flasks were used for one analysis, both containing 4 ml. of plasma and 0·3 ml. of 3 M-acetate buffer pH 4·9 in the main compartment and one containing 0·5 ml. of washed bacterial cells in the side arm.

## RESULTS

*Glutamine and glutamic acid in animal tissues.* Results obtained on various tissues, excluding blood and foetal tissues, are shown in Table 1. All tissues examined contained considerable quantities of glutamic acid and in most glutamine was also present. The sum of glutamic acid and glutamine in different samples of the same tissue varied less from animal to animal than the concentrations of the two components. In most tissues the concentration of glutamic acid + glutamine was much greater (*c.* 10 times) than the concentrations in the blood plasma, exceptions being adipose tissue, the crystalline lens and vitreous humour, all tissues whose metabolic activities are in general low. The highest concentrations were found in brain, mammalian heart and spleen (average concentrations between 10 and $15 \times 10^{-6}$ mol./g. or 146–220 mg./100 g.). Relatively low values (average below $5 \times 10^{-6}$ mol./g. or 73 mg./100 g.) were found in ovary, thyroid, lung and the tissues already given as having concentrations of the same order as blood plasma. Intermediate values (average between 5 and $10 \times 10^{-6}$ mol./g. or 73–146 mg./100 g.) were found in the other tissues tested.

The proportion of glutamic acid to glutamine showed consistent differences from tissue to tissue. The mammalian heart was the only material in which glutamine regularly constituted the major part. In all other tissues examined including avian heart, glutamic acid usually predominated, though in varying degrees. In the spleen the ratio glutamic acid/glutamine was 6–10, in brain about 2, in most other tissues variable.

In foetal tissues (Table 2) the sum of glutamic acid and glutamine was generally lower than in the adult tissues, an exception being the lung. The ratio glutamic acid/glutamine was higher in most foetal tissues than in the adult tissue, especially in the heart. Among the foetal tissues thymus showed the highest concentration of glutamic acid.

*Human blood plasma.* Fifty-four specimens from fasting hospital patients were examined (Table 3). In all normal cases the average content of glutamic acid was 3·47 mg./100 ml. and of glutamine 5·78 mg./100 ml. No major deviations were found in the forty-three pathological specimens. In the group 'infectious diseases', the glutamic acid values seemed to be higher, and the glutamine values lower than in the other groups, but in view of the small number of cases no definite conclusions can be drawn. As in other materials, the concentration of glutamic acid + glutamine showed much less variation than did the concentrations of the two components separately. The average sum of all cases was 8·74 mg./100 ml., of which 42·6 % was glutamic acid and 57·4 % glutamine. The data are in general

Table 1. *Glutamic acid, glutamine and ammonia in animal tissues*

| Tissue | Animal | Amounts of substance found ($10^{-6}$ mol./g.) Glutamic acid | Glutamine | Glutamic acid +glutamine | Ammonia |
|---|---|---|---|---|---|
| Liver | Sheep | 5·45 | 2·27 | 7·72 | 2·68 |
| | Sheep | 6·53 | 0 | 6·53 | 2·08 |
| | Sheep | 5·47 | 2·66 | 8·13 | 1·21 |
| | Cat | 2·86 | 3·66 | 6·52 | 4·08 |
| | Pigeon | 6·46 | 0 | 6·46 | 2·68 |
| | Pigeon | 5·53 | 5·93 | 11·46 | 2·50 |
| | Pigeon | 5·75 | 5·35 | 11·10 | 6·15 |
| Spleen | Sheep | 10·60 | 1·47 | 12·07 | 3·39 |
| | Sheep | 9·59 | 1·56 | 11·15 | 3·16 |
| | Sheep | 10·95 | 1·01 | 11·96 | 3·18 |
| | Cat | 9·55 | 1·38 | 10·93 | 3·39 |
| Kidney cortex | Sheep | 7·90 | 1·21 | 9·11 | 2·16 |
| | Sheep | 4·86 | 0 | 4·86 | 4·24 |
| | Sheep | 5·95 | 0·87 | 6·82 | 4·17 |
| | Cat | 9·41 | 1·12 | 10·53 | 4·55 |
| Kidney medulla | Sheep | 7·00 | 1·79 | 8·79 | 1·96 |
| | Sheep | 4·23 | 2·08 | 6·31 | 1·16 |
| | Sheep | 7·08 | 0 | 7·08 | 4·59 |
| | Cat | 5·30 | 3·70 | 9·00 | 4·19 |
| Brain, grey matter | Sheep | 11·1 | 4·19 | 15·29 | 2·71 |
| | Sheep | 9·93 | 3·36 | 13·29 | 4·39 |
| Brain, white matter | Sheep | 7·01 | 3·36 | 10·37 | 1·56 |
| | Sheep | 5·30 | 3·28 | 8·58 | 2·88 |
| Brain, whole | Cat | 9·93 | 5·27 | 15·20 | 2·05 |
| | Pigeon | 6·16 | 6·99 | 13·15 | 0·93 |
| | Pigeon | 13·95 | 4·52 | 18·47 | 5·71 |
| | Pigeon | 8·25 | 5·75 | 14·00 | 2·88 |
| | Pigeon | 10·28 | 5·60 | 15·88 | 6·06 |
| Lung | Sheep | 2·38 | 1·54 | 3·92 | 2·08 |
| | Sheep | 3·67 | 0 | 2·67 | 3·74 |
| | Sheep | 4·15 | 0·73 | 4·88 | 0·94 |
| | Cat | 5·17 | 1·85 | 7·02 | 6·37 |
| | Pigeon | 1·67 | 2·14 | 3·81 | 4·08 |
| | Pigeon | 3·19 | 0·80 | 3·99 | 4·77 |
| Heart | Sheep | 2·99 | 14·0 | 16·99 | 1·79 |
| | Sheep | 2·09 | 11·2 | 13·29 | 1·87 |
| | Sheep | 1·34 | 9·25 | 10·59 | 1·74 |
| | Cat | 5·17 | 9·89 | 15·06 | 8·37 |
| | Pigeon | 3·13 | 5·41 | 8·54 | 4·95 |
| | Pigeon | 7·80 | 2·05 | 9·85 | 2·08 |
| | Pigeon | 6·34 | 3·64 | 9·98 | 6·25 |
| Pancreas | Sheep | 4·87 | 1·94 | 6·81 | 2·05 |
| | Sheep | 3·01 | 0 | 3·01 | 3·14 |
| | Sheep | 5·66 | 2·41 | 8·07 | 3·39 |
| | Pigeon | 5·53 | 7·54 | 13·07 | 7·83 |
| | Pigeon | 9·56 | 3·14 | 12·70 | 7·20 |
| Skeletal muscle | Sheep | 5·58 | 2·41 | 7·99 | 2·50 |
| | Sheep | 6·73 | 7·08 | 13·81 | 2·08 |
| | Cat | 0·74 | 5·55 | 6·29 | 6·37 |
| | Pigeon | 0 | 3·28 | 3·28 | 7·70 |
| | Pigeon | 8·15 | 1·03 | 9·18 | 5·08 |
| | Pigeon | 3·30 | 2·48 | 5·78 | 6·81 |
| Smooth muscle, gizzard | Pigeon | 10·52 | 3·56 | 14·08 | 2·72 |
| | Pigeon | 6·69 | 1·78 | 8·47 | 2·61 |
| | Pigeon | 2·72 | 3·90 | 6·62 | 4·91 |
| Testis | Sheep | 4·28 | 4·48 | 8·76 | 2·48 |
| | Sheep | 7·82 | 2·14 | 9·96 | 3·92 |

Table 1 (*cont.*)

| Tissue | Animal | Amounts of substance found ($10^{-6}$ mol./g.) Glutamic acid | Glutamine | Glutamic acid + glutamine | Ammonia |
|---|---|---|---|---|---|
| Ovary | Sheep | 1·32 | 1·63 | 2·95 | 1·36 |
| | Sheep | 4·21 | 0·58 | 4·79 | 1·94 |
| Suprarenal gland (mainly cortex) | Sheep | 3·55 | 1·52 | 5·07 | 4·91 |
| | Cow | 4·35 | 0·74 | 5·09 | 1·96 |
| Thyroid gland | Sheep | 0·80 | 0·47 | 1·27 | 3·52 |
| | Cow | 2·28 | 0 | 2·28 | 1·61 |
| | Cow | 4·28 | 0 | 4·28 | 2·68 |
| Lymph gland | Sheep | 6·09 | 2·28 | 8·37 | 4·35 |
| | Cow | 9·18 | 2·27 | 11·45 | 3·01 |
| | Cow | 6·60 | 1·65 | 8·25 | 0·40 |
| Gastric mucosa | Sheep | 2·70 | 0·94 | 3·64 | 3·18 |
| Duodenal mucosa | Sheep | 5·44 | 1·21 | 6·65 | 5·42 |
| | Cat | 5·03 | 3·60 | 8·63 | 6·34 |
| Vitreous humour | Sheep | 0·87 | 0 | 0·87 | 1·36 |
| Lens | Sheep | 1·07 | 0·58 | 1·65 | 0·67 |
| Fat, peritoneal | Sheep | 0·62 | 0·45 | 1·07 | 1·23 |

Table 2. *Glutamic acid, glutamine and ammonia in foetal tissues and placenta*

| Tissue | Animal | Approx. age of foetus (weeks) | Amounts of substance found ($10^{-6}$ mol./g.) Glutamic acid | Glutamine | Glutamic acid + glutamine | Ammonia |
|---|---|---|---|---|---|---|
| Liver | Calf | 10 | 3·78 | 1·03 | 4·81 | 1·79 |
| | Calf | 30 | 2·74 | 3·90 | 6·64 | 1·47 |
| | Sheep | 6–7 | 3·68 | 1·87 | 5·55 | 4·42 |
| Brain (whole) | Calf | 10 | 4·76 | 1·05 | 5·81 | 1·56 |
| | Calf | 30 | 8·16 | 2·61 | 10·77 | 2·07 |
| Brain | Sheep | 6–7 | 2·34 | 2·28 | 4·62 | 1·87 |
| Kidney | Calf | 10 | 5·55 | 0 | 5·55 | 1·47 |
| | Calf | 30 | 4·82 | 0·62 | 5·44 | 2·03 |
| Heart | Calf | 10 | 7·85 | 2·49 | 10·34 | 1·16 |
| | Calf | 30 | 4·02 | 6·36 | 10·38 | 2·54 |
| | Sheep | 6–7 | 0·49 | 0 | 0·49 | 5·90 |
| Lung | Calf | 10 | 7·28 | 0·89 | 8·17 | 0·94 |
| Thymus | Calf | 10 | 9·85 | 0·94 | 10·79 | 5·34 |
| | Calf | 30 | 11·30 | 0·89 | 12·19 | 2·81 |
| Placenta | Calf | 10 | 7·30 | 1·92 | 9·22 | 1·63 |
| | Calf | 30 | 6·76 | 2·03 | 8·79 | 1·94 |
| | Sheep | 6–7 | 2·45 | 2·05 | 4·50 | 4·86 |
| | Cat | Almost full term | 2·54 | 5·17 | 7·71 | 1·87 |
| Spleen | Calf | 30 | 6·96 | 0·60 | 7·56 | 2·52 |
| Bone marrow, femur | Calf | 30 | 2·01 | 1·74 | 3·75 | 2·94 |

agreement with the glutamine determinations in blood plasma published by Harris (1943), Archibald (1944) and Prescott & Waelsch (1947), though the present average values are a little lower. The average values for glutamic acid, in contrast, are somewhat higher than those reported by Prescott & Waelsch.

## DISCUSSION

*Glutamic acid and glutamine in animal tissues.* The literature does not contain many data which are comparable with those presented in this paper. Ferdman *et al.* (1942) examined the readily hydrolyzable 'amide nitrogen' in various tissues of the dog, cat, rabbit, pigeon and horse; the fresh tissue was frozen in liquid air, extracted with trichloroacetic acid and the increase of ammonia formed on hydrolysis (5–10 min.; 100°; 5 % sulphuric acid) was determined. Hamilton (1945) heated the picric acid extract of dog tissues to 100° at pH 6·5 for 90 min., and estimated the decrease in 'carboxyl nitrogen' by the ninhydrin method of Van Slyke, Dillon, MacFadyen & Hamilton (1941). The figures obtained by Ferdman *et al.* (1942) and Hamilton (1945) are of the same order as those reported in the present paper.

Table 3. *Glutamine and glutamic acid in human blood plasma*

(The results are expressed, in accordance with the practice of previous authors, as mg./100 ml. plasma. Glutamine is expressed as glutamic acid.)

| | Plasma (mg./100 ml.) | | |
|---|---|---|---|
| | Range | Mean | S.D. |
| Normal (11 cases): | | | |
| Glutamic acid | 1·3– 5·9 | 3·41 | 1·39 |
| Glutamine | 2·7– 7·8 | 5·78 | 1·55 |
| Total | 7·6–10·1 | 9·19 | 0·84 |
| Diseases of the circulatory system (13 cases): | | | |
| Glutamic acid | 1·7– 5·3 | 3·74 | 1·13 |
| Glutamine | 3·5– 8·0 | 5·60 | 1·18 |
| Total | 6·2–10·6 | 9·34 | 1·17 |
| Malignant tumours (5 cases): | | | |
| Glutamic acid | ·1·4– 4·6 | 2·94 | 1·12 |
| Glutamine | 2·6– 7·6 | 5·02 | 2·08 |
| Total | 7·2– 9·8 | 7·96 | 1·07 |
| Infectious diseases (12 cases): | | | |
| Glutamic acid | 2·4– 6·6 | 4·17 | 1·42 |
| Glutamine | 2·0– 6·8 | 4·05 | 1·58 |
| Total | 6·3–10·2 | 8·22 | 1·27 |
| Miscellaneous (13 cases): | | | |
| Glutamic acid | 0* – 7·2 | 3·96 | 2·14 |
| Glutamine | 2·0– 9·2 | 4·64 | 2·27 |
| Total | 5·6–11·4 | 8·60 | 2·11 |

* No glutamine was present in one case of thyrotoxicosis (basal metabolic rate +29 %).

Hamilton (1945) has already pointed out that in cardiac muscle of the dog glutamine contributes 50–60 % of the free total 'carboxyl nitrogen' of the tissue. A comparison of the present data with estimations of the total amino nitrogen in animal tissues (Van Slyke, 1913; Hamilton, 1945) indicates that in most tissues the sum of glutamic acid and glutamine represents 25–60 % of the total amino nitrogen.

Van Slyke (1913) was the first to note that tissues contain 5–10 times more amino nitrogen than blood plasma. Hamilton found a similar proportion for glutamine. The present data show that this is also true for glutamic acid.

The question may be raised whether the glutamic acid found in tissue suspension has, wholly or in part, arisen from glutamine after death, as a result of the action of glutaminase. The ammonia values given in Tables 1 and 2 are in most tissues much lower than the glutamic acid values. This applies especially to the tissues which are known to contain a glutaminase (liver, kidney, brain). Most of the glutamic acid found in these tissues must, therefore, have been preformed.

*Ammonia in animal tissues.* Many data are available on the ammonia content of blood and of other body liquids, but of the tissues only cardiac and striated muscle have been thoroughly studied, mainly by the schools of Parnas and Embden. As Parnas (1928) has pointed out, it is uncertain whether ammonia found in animal tissues, even in material treated with liquid air, is preformed or arises after death. This has to be borne in mind in the interpretation of results.

## SUMMARY

1. The decarboxylase method (Gale, 1945; Krebs, 1948) has been used to determine separately glutamic acid and glutamine in twenty-four different animal tissues (including foetal material), and in fifty-four specimens of human blood plasma.

2. The sum of the concentrations of glutamic acid and glutamine was highest in brain cortex, heart, spleen and thymus (average $10–15 \times 10^{-6}$ mol./g.). Average values below $5 \times 10^{-6}$ mol./g. were found in ovary, thyroid and lung, and below $1 \times 10^{-6}$ mol./g. in most other tissues.

3. In the mammalian heart and in blood plasma glutamine was present in greater quantities than glutamic acid. In other tissues glutamic acid predominated as a rule.

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# A Method for Determining the Sedimentation Constant of Material of Low Molecular Weight: Studies on Oxidation Products of Insulin


By H. GUTFREUND, *Department of Colloid Science, University of Cambridge*
and A. G. OGSTON, *Department of Biochemistry, University of Oxford*


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In a recent paper one of us (Gutfreund, 1948) has shown that insulin molecules probably consist of subunits of molecular weight 12,000; these units polymerize to molecules of weight 48,000 in neutral solutions of moderate insulin concentration (0·5–1%). The mean molecular weight is, however, dependent upon pH, temperature and concentration. From the determination of terminal amino groups of insulin, Sanger (1945) suggested that these submolecules, of molecular weight 12,000, are made up of four peptide chains bound together by disulphide linkages. Two of these chains have terminal glycyl residues and two have terminal phenylalanyl residues. Sanger (1947) has shown that the disulphide linkages can be split by oxidation with performic acid, without affecting any other part of the insulin molecule. Some preliminary studies on the peptides resulting from this oxidation of insulin have been reported by Sanger (1947).

It was the purpose of the work described in this paper to develop methods for the determination of sedimentation constants ($S_{20}$) less than $1 \times 10^{-13}$, and to study fractions from oxidized insulin both by ultracentrifugal sedimentation and by diffusion. Sanger (1947) suggested that the peptides obtained on oxidation of insulin should have a molecular weight of about 3000. This value is between the ranges of molecular weights which have been studied by methods suitable for macromolecules (osmotic pressure, sedimentation and diffusion, etc.) on the one hand, and those used for simpler compounds (freezing-point depression and similar procedures) on the other hand. It was necessary to modify the procedure of computing sedimentation constants to make it useful for the purpose of studying these polypeptides.

## EXPERIMENTAL

Ultracentrifugal examinations were carried out in a Svedberg oil turbine ultracentrifuge, and the Philpot (1938) optical system was used for the observation of the boundaries. The speed of the centrifuge was about 1010 r.p.sec. Diffusion constants were determined by the method of Coulson, Cox, Ogston & Philpot (1948).

Dr F. Sanger kindly prepared for us all the oxidized insulin and fractions thereof used in this work. The material was prepared and fractionated as described by Sanger (1949). Crystalline zinc insulin (obtained from Boots Pure Drug Co. Ltd.) was used as starting material. Two fractions (*A* and *B*) were examined. Fraction *A* contains the peptides with terminal glycyl residues while fraction *B* contains those with terminal phenylalanyl residues. The purity of each fraction, as shown by end-group assay, was about 95%.

It was found that oxidized insulin diffused slowly through collodion or cellophan membranes on dialysis; as it was essential to get the solutions of oxidized insulin into equilibrium with a salt solution of known composition, these were dialyzed against a large volume of M-$Na_2HPO_4$. Up to half the nitrogenous material was lost from the solutions.

*Method of calculation of the sedimentation constant in the absence of a clear boundary of sedimentation*

Two methods are in general use for the observation of sedimentation constants of proteins; first the light absorption method, giving a direct plot of concentration $c$ against distance $x$ in the cell, and secondly the refractive index method, giving a plot of concentration gradient $dc/dx$ against $x$. Whichever method is used, the sedimentation constant is normally calculated from the movement of the mid-point of the protein boundary with time. This procedure is accurate only if the calculations are made from measurements on boundaries which have completely left the meniscus of the solution and have not reached the bottom of the cell. The peptides studied during this investigation have such small sedimentation and high diffusion constants that one end of the boundary is at the bottom of the cell by the time the mid-point (or peak) has left the meniscus, and it is, therefore, impossible to get successive pictures of the whole boundary (see Fig. 1).

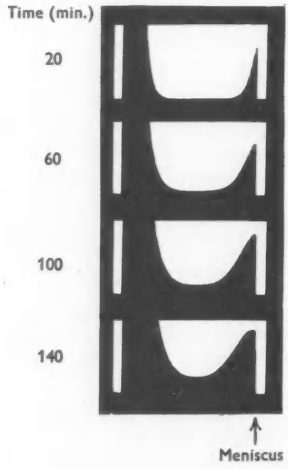


Fig. 1. Philpot schlieren diagrams of fraction *A* 20, 60, 100 and 140 min. after full speed had been attained. Oxidized insulin concentration 0·74%.

It has been suggested by Tiselius, Pedersen & Svedberg (1937) that, in such cases, the movement of the quantity of protein across an arbitrary plane in the cell could be used for the calculation of the sedimentation constant. This method was elaborated by Svedberg & Pedersen (1940), but no practical use appears to have been made of it so far, and no working procedure has been described; we have, therefore, developed the following method for the calculation of the sedimentation constant of very slowly sedimenting substances from photographs obtained by the Philpot (1938) optical method.

Fig. 2 is a diagram of the ultracentrifuge cell, with a plot of the concentration and of the gradient of concentration. A fixed plane, distant $X$ cm. from the centre of rotation, is chosen so that the concentration in its neighbourhood varies only with time $t$

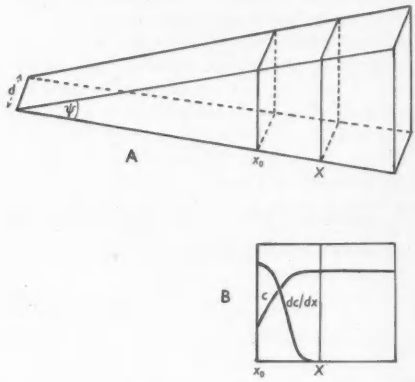


Fig. 2. A, diagram of the ultracentrifuge cell showing the dimensions used in the calculation; B, plots of $c$ and $dc/dx$ against $x$.

and not with the distance $x$ from the centre of rotation. The sedimentation constant can be obtained from the rate at which material passes the plane $X$. The concentration at $X$ (and throughout the cell) is initially $c_0$, but after time $t$ (assuming that the speed of rotation $\omega$ and the sedimentation constant $s$ do not vary) it has fallen to

$$c_{x,t} = c_0 \, e^{-2s\omega^2 t}. \qquad (1)$$

The rate at which material is passing $X$ at time $t$ is, therefore,

$$\frac{dQ}{dt} = c_{x,t} \, s\omega^2 X \, \psi X d \, . \, dt$$
$$= \psi d \, c_0 \, s\omega^2 X^2 \, e^{-2s\omega^2 t} \, dt.$$

Integrating between 0 and $t$, the amount of material which has passed across $X$ from the upper region of the cell is

$$\Delta Q_t = \psi d \, . \, \frac{c_0 X^2}{2} \, (1 - e^{-2s\omega^2 t}). \qquad (2)$$

This quantity $\Delta Q_t$ can be obtained from the schlieren diagrams. The concentration at $x$, $t$ is given by

$$c_{x,t} = c_{X,t} - \int_x^X \frac{dc}{dx} \, dx. \qquad (3)$$

The quantity of material above $X$ at time $t$ is then given by

$$Q_t = \int_{x_0}^{X} c_{x,t}\, \psi x d\,.\,dx$$

which, from (1) and (3),

$$= \psi d \left\{ \int_{x_0}^{X} c_0\, e^{-2s\omega^2 t}\,.\,x\, dx - \int_{x_0}^{X} x \int_{x}^{X} \frac{dc}{dx}\, dx.\, dx \right\}$$

$$= \psi d \left\{ c_0\, (X^2 - x_0^2)\, e^{-2s\omega^2 t}/2 - \int_{x_0}^{X} x \int_{x}^{X} \frac{dc}{dx}\, dx\,.\,dx \right\}. \quad (4)$$

The quantity initially above $X$ was

$$Q_0 = \int_{x_0}^{X} c_0\, \psi x d\,.\,dx = \psi d\,.\,c_0\, (X^2 - x_0^2)/2. \quad (5)$$

Since $\Delta Q_t = Q_0 - Q_t$ and writing $Q_t'$ for the second term in the bracket of (4),

$$c_0\, X^2\, (1 - e^{-2s\omega^2 t})/2 = c_0\, (X^2 - x_0^2)/2 - c_0\, (X^2 - x_0^2)\, e^{-2s\omega^2 t}/2 + Q_t',$$

whence $$Q_t' = c_0\, x_0^2\, (1 - e^{-2s\omega^2 t})/2.$$

This expression is exact. It can be solved, without further approximation, by converting it into the form

$$\log \left\{ 1 - \frac{2Q_t'}{c_0 x_0^2} \right\} = -\frac{2s\omega^2}{2{\cdot}303}\, t. \quad (6a)$$

By plotting the log term against $t$, a straight line of slope $-2s\omega^2/2{\cdot}303$ should be obtained. Where $s\omega^2 t$ is small, equation ($6a$) approximates to

$$Q_t' = c_0 x_0^2\, s\omega^2 t. \quad (6b)$$

This method of solution amounts to the assessment of the amount of material which would have passed the plane at $x_0$ had the solution not been terminated there by the meniscus; this is measured by the amount of material which is absent below $x_0$ compared with expectation, making allowance for the 'dilution factor'.

*Computation*

Enlarged projections ($\times 4{\cdot}5$) of the boundary photographs were traced on to graph paper; the base lines were fitted from the corresponding photographs of a control run done on buffer alone. Values of $\int_x^X \frac{dc}{dx}\, dx$ were obtained for a series of values of $x$ in each tracing by counting squares, and were converted into units of refractive increment by use of the appropriate optical constants. Each value was then multiplied by the corresponding value of $x$ (the actual distance from the centre of rotation) and each product $x \int_x^X \frac{dc}{dx}\, dx$ was plotted against $x$. Graphical integration of each resulting curve between $x_0$ and $X$ gave the value of $Q_t'$.

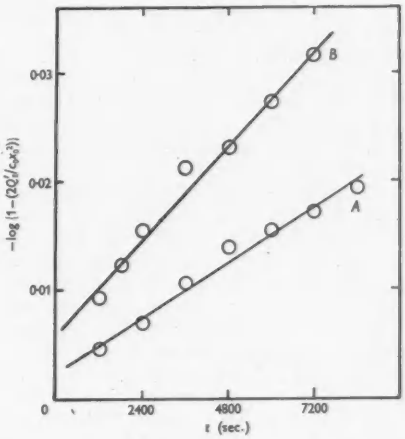


Fig. 3. Plot of $-\log \left\{ 1 - \frac{2Q_t'}{c_0 x_0^2} \right\}$ (in terms of refractive index) against time of sedimentation $t$ from reaching full speed, for fractions $A$ and $B$.

Fig. 3 shows the values of $-\log\,(1 - (2Q_t'/c_0 x_0^2)$ plotted against $t$ for the two fractions of oxidized insulin. The variations of $\omega^2$ and of the temperature together affect the rate of sedimentation by less than 1 % and their mean values have therefore been used; the regression coefficient $b$ and the standard deviation of $b$ have been calculated in each case. The sedimentation constant $s$ is then given by

$$s = 2{\cdot}303\, b/2\omega^2.$$

The sedimentation constant in water at 20°, $S_{20}$ (corr.), is obtained by applying the usual corrections to $s$, including that for error of reading of the cell temperature (Cecil & Ogston, 1949).

## RESULTS

The values of $S_{20}$ (corr.) and of $D_{20}$ (corr.) (measured at the same concentrations) are given in Table 1; the molecular weights have been obtained from these values, assuming a value of 0·75 for the partial specific volume.

Table 1. *Sedimentation and diffusion constants of oxidized insulin fractions*

| | $S_{20}$ (corr.) $\times 10^{13}$ | S.D. of $S_{20}$ (corr.) $\times 10^{13}$ | $D_{20}$ (corr.) $\times 10^{7}$ | Molecular wt. |
|---|---|---|---|---|
| Fraction *A* | 0·53 | 0·034 | 17·6 | 2900 |
| Fraction *B* | 0·91 | 0·054 | 12·6 | 7000 |

## DISCUSSION

The diffusion results indicated that fraction *A* was nearly homogeneous, whereas fraction *B* was heterogeneous. The method of preparation would also suggest that fraction *A* is the more homogeneous, fraction *B* probably containing a certain amount of incompletely oxidized insulin.

The simplest assumption from Sanger's (1945) data is that the unit of insulin is split by oxidation into four peptide chains, two of these having glycyl end-groups (fraction *A*) and the other two having phenylalanyl end groups (fraction *B*). The molecular weight estimated for the former agrees moderately with Sanger's (1947) estimate of 2500 from analytical data. By difference from the total weight of 12,000, the molecular weight of fraction *B* should then be about 3100; the discrepancy between this and the value estimated suggests that fraction *B* contains a considerable proportion of incompletely oxidized material, which would seriously affect the values of both sedimentation and diffusion constants.

The main purpose of this paper was to describe a general method for studying these polypeptides in the ultracentrifuge; the results of the examination of the oxidation products of insulin are necessarily only preliminary. It is possible that some fraction of much smaller molecular weight was lost during dialysis and the assumption of two chains with glycyl end groups and two with phenylalanyl end groups, making up the unit of molecular weight 12,000, may well be an oversimplification. The increase in molecular weight on oxidation (600 per 12,000) has not been taken into consideration in the above calculation.

## SUMMARY

1. Details are given of a method of obtaining the sedimentation constant of material of too small a molecular weight to form a clear sedimenting boundary in the ultracentrifuge.

2. This method has been applied to the study of the peptides obtained by partial degradation of insulin with performic acid. The diffusion constants of two fractions have also been measured and estimates made of their molecular weights.

One of us (H. G.) is indebted to Prof. R. A. Peters, F.R.S., for hospitality in his laboratory.

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# Protease Inhibitors

## 1. ASSAY AND NATURE OF SERUM ANTIPROTEASE

By E. S. DUTHIE and L. LORENZ, *Lister Institute, Elstree, Herts*

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The inhibition of trypsin by normal serum has been studied by many workers (Landsteiner, 1900; Fujimoto, 1918; Hussey & Northrop, 1922–3; Grob, 1943). The association of the trypsin inhibitor with the albumin fraction first noted by Landsteiner (1900) has been confirmed by subsequent workers using salt fractionation, and Smith & Lindsley (1939) have shown that on electrophoretic separation of the serum proteins the antitryptic power lay mostly in the albumin fraction. Several groups of workers (Schwartz, 1909; Bauer, 1910; Jobling & Petersen, 1914) have associated antitryptic activity in serum with lipids or fatty acids, and Ungar (1945) has found that it was removed by ether extraction and reconstituted by readdition of the ether extract to the aqueous residue. This association of antitryptic activity with lipids has been denied by many others (Meyer, 1909; Cobliner, 1910; Teale & Bach, 1919–20).

Studies by Christensen & MacLeod (1945) and MacFarlane & Pilling (1946) show that plasma trypsin (plasmin) is likewise inhibited by a constituent of the albumin fraction of plasma which the latter authors term antiplasmin. Beloff (1946) has shown that the skin protease studied by Beloff & Peters (1944, 1945, 1946) is also inhibited by the albumin fraction of serum. Her work clearly demonstrates that the inhibitory factor against skin protease is identical with the inhibiting factor for both trypsin and plasma trypsin, although she drew the opposite conclusion from a study of the literature. Schmitz (1937, 1938) claims to have isolated from serum a second antitryptic factor which he regards as a polypeptide similar in properties to that isolated from pancreas by Kunitz & Northrop (1936), and which he believes is normally present in combination with plasma trypsin, thereby inactivating it. The evidence brought forward by Schmitz is hardly sufficient to support his claims. Grob (1943) appears to have prepared a 'crystalline' serum antitrypsin by the methods of Schmitz. Many authors have described marked physiological and pathological variations in the antitrypsin activity of the sera of man and animals and a good summary is given by Grob (1943). The majority of the conditions reported showed a marked increase over the normal level, but no single factor appears to be responsible, and the work is difficult to assess because of the wide variation in methods used, and the lack of any simple accurate technique for assaying serum antitrypsin.

The development by Swyer & Emmens (1947) of a satisfactory viscosimetric relationship (in the case of hyaluronidase) between enzyme activity and flow time, as measured in Ostwald viscosimeters, made it possible to assay serum antitrypsin viscosimetrically using gelatin as substrate and to restudy some of the findings of previous workers.

## EXPERIMENTAL

### *Materials and methods*

*Gelatin solution.* Gelatin 10% (w/v) in 0·85% (w/v) NaCl (saline) was autoclaved and stored in the cold in 100 ml. amounts. After warming, 10 ml. of 0·2 M-phosphate buffer (pH 7·6) were added to every 90 ml. gelatin, and the mixture was then brought to pH 8·0 by addition of a few drops of 2 N-NaOH. The solution was heated to nearly 100° and filtered through a Whatman no. 1 filter paper, the pH was adjusted to 7·6, and saline added so that the final relative viscosity after dilution with an equal volume of 0·5% (w/v) gum acacia in saline was about 2·3. The gelatin was stored in the cold until next day when it was melted at 37·5° and kept at that temperature during use. Merthiolate was added as a preservative.

*Trypsin solution.* Crystalline trypsin containing approximately 50% (w/v) $MgSO_4$ was dissolved at 0·1% (w/v) concentration in 0·01 N-HCl and stored at 4°. Dilutions were made in 0·5% (w/v) gum acacia. As a standard of comparison, the enzyme was assigned the arbitrary value of 20,000 units/mg.

*Estimation of enzyme and inhibitor.* Equal volumes of enzyme and gelatin solution were mixed at 37°, and 2 ml. of the mixture transferred to Ostwald viscosimeters in a water bath at 37·5°. The viscosity of the mixture was measured 20 min. after mixing, and this was carried out more accurately by taking readings at 16, 20 and 24 min. respectively and interpolating when necessary. Mixtures of inhibitor and enzyme were incubated for 10 min. at 37° prior to mixing with gelatin. The viscosimeters used had a bulb capacity of a little more than 1 ml., a capillary of 0·5 mm. diam. and a flow time of $51 \pm 3$ sec.

### *Viscosimetric assay of enzyme and inhibitor*

*Enzyme.* Although several investigators (Hussey & Northrop, 1922–3; Northrop & Kunitz, 1932–3; Smith & Lindsley, 1939; Christensen, 1945) have used viscosimetric

methods for the assay of proteolytic enzymes using gelatin as a substrate, it is doubtful if any of the formulae developed, which relate enzyme concentration to a rate of fall in viscosity, are entirely satisfactory. Swyer & Emmens (1947), in a study of hyaluronidase action, defined the flow-time index $v$ as

$$\frac{(f_s - f_e)}{(f_s - f_o)} \times 1000,$$

where $f_s$ = flow time of substrate and enzyme solution; $f_o$ = flow time of solvents alone (these being in the same proportion as in the hydrolysis mixture); and $f_e$ = the flow time of the enzyme substrate mixture after incubation for a given time such as 20 min. They showed that $v$ gives a straight-line relationship when plotted against the logarithm of the enzyme concentration.

Since $f_s - f_o$ is the viscosity due to the substrate, and $f_s - f_e$ represents the reduction of viscosity caused by the action of the enzyme on the substrate, and since the curve relating flow time to substrate concentration is approximately linear at the concentrations of hyaluronic acid used,

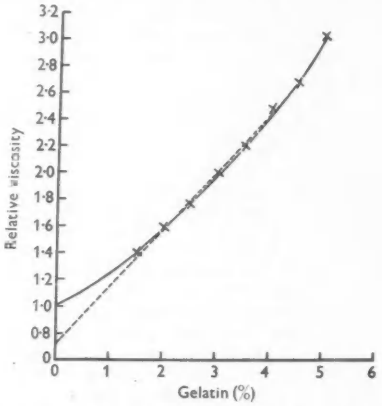


Fig. 1. Relationship between concentration of gelatin and relative viscosity as determined in an Ostwald viscosimeter.

their finding evidently rests on the assumption that the flow-time index $v$ is a measure of the fraction of the total substrate hydrolyzed in a given time. Between relative viscosities 2·7 and 1·6 the curve relating relative viscosity (or flow time) to gelatin concentration is likewise approximately linear (Fig. 1). The line does not pass through viscosity 1·0 as it would if a lower range of concentration were considered, but through a value, in this case 0·76, which depended on the batch of gelatin used and showed little tendency to vary. Working between initial and final relative viscosities of 2·5 and 1·6, an approximately linear relationship (Fig. 2) was found when $v$, the flow-time index, was plotted against the logarithm of the enzyme concentration for values of $v$ between 150 and 730. The flow-time index can, therefore, be used as a measure of the amount of substrate hydrolyzed within a given range of viscosities, provided that these viscosities give a straight line when plotted against gelatin concentration and irrespective of where the line cuts the ordinate.

Gelatin as used in the assays increased slightly in viscosity during the first 3 hr. incubation at 37·5°, after which it underwent a slow loss amounting to between 0·01 and 0·02 unit relative viscosity/hr. Neither this slight fall nor the day-to-day variations encountered in making up the solutions had any significant effect on the value of $v$ obtained provided the $f_s$ values were redetermined at intervals.

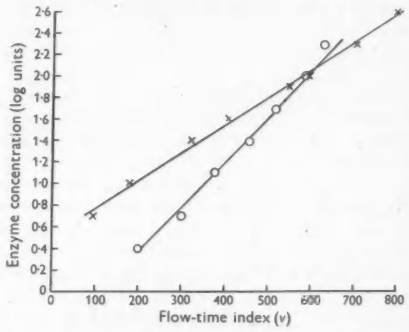


Fig. 2. Relationship between flow-time index ($v$) and logarithm of enzyme concentration for crystalline trypsin and crystalline chymotrypsin. ×—× Crystalline trypsin; ○—○ crystalline chymotrypsin.

Table 1 shows that even large variations in the initial viscosity of the gelatin from 2·5 to 1·96 altered $v$ by only 40, there being a rise of about 7·4 in the flow-time index for each fall of 0·1 in the relative viscosity of the gelatin. In practice it was sufficient to adjust the viscosity each day to the same value, and to redetermine it at intervals on one

Table 1. *Effect of change in initial viscosity of gelatin on the flow-time index v obtained with crystalline trypsin: each value an average of four to six determinations*

| Relative viscosity | $v$ | |
|---|---|---|
| | Trypsin (100 units) | Trypsin (25 units) |
| 2·50 | 563 | 263 |
| 2·20 | 590 | 288 |
| 1·96 | 604 | 304 |

viscosimeter, since the slope of the line relating the logarithm of the enzyme concentration to the flow-time index was identical at different viscosities, and for many months the flow-time index for 100 units of crystalline trypsin was constant at about 600. Slightly different values were obtained with other samples of gelatin. The slope of the line obtained with crude trypsin was identical, but that of crystalline chymotrypsin (Fig. 2) was different.

## RESULTS

*Rate of reaction of the inhibitor on enzyme.* The inhibition of crystalline trypsin by serum increases with the time of contact before the addition of

substrate (Fig. 3), the main part of the reaction being completed in the first 20 min. The 10 min. incubation period used in the course of the work was based on preliminary trials with crude trypsin, which showed very little increase in inhibition by serum after this time.

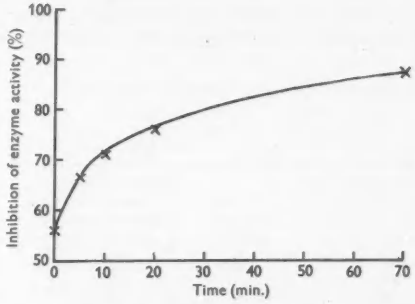


Fig. 3. Effect of time of contact of serum and trypsin solution at 37° before mixing with gelatin on the inhibition of enzyme.

*Effect of concentration of inhibitor on degree of inhibition.* The inhibitory action of varying concentrations of serum on given concentrations of crude and crystalline trypsin of equal activity is shown in Fig. 4, where the residual activity as

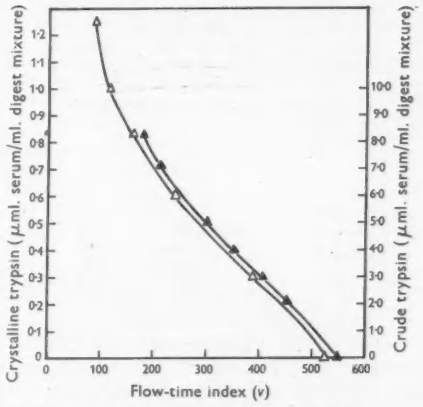


Fig. 4. Effect of addition of varying amounts of serum on flow-time index of 75 units/ml. of crude and crystalline trypsin (1 mg. crystalline trypsin = 20,000 units). △—△, crystalline trypsin + guinea pig serum; ▲—▲, crude trypsin + horse serum.

measured by $v$ is plotted against volume of serum added. Apart from the fact that some ten times more serum was required to produce a comparable degree of inhibition in the case of crude trypsin, the curves are identical. When the percentage inhibition of enzyme activity at each serum level is calculated by a comparison of Figs. 2 and 4, and this is plotted against the serum volumes added, a curve is obtained (Fig. 5) which is very similar to that obtained by

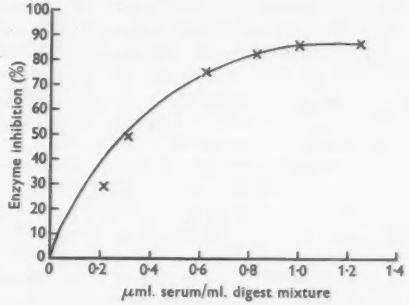


Fig. 5. Effect of increasing amounts of serum on a constant amount of enzyme (75 units/ml.) expressed as per cent enzyme inhibition (1 mg. crystalline trypsin = 20,000 units).

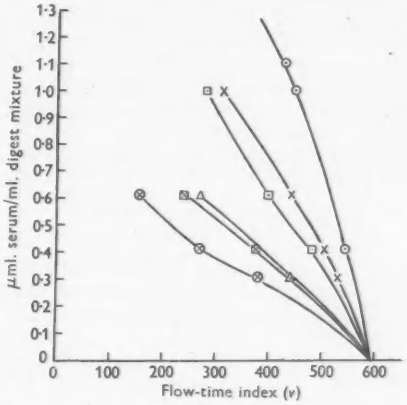


Fig. 6. Inhibition of trypsin by various sera as measured by the flow-time index; ⊙, rabbit; ⊡, horse; △, sheep; ×, normal human; ⊠, human (pregnancy); ⊗, human (tuberculosis).

Hussey & Northrop (1922–3), who studied the action of serum on crude trypsin, and of Kunitz (1947), who investigated the effect of soya-bean trypsin inhibitor on crystalline chymotrypsin. Kunitz has derived an equation, relating the degree of inhibition and the amount of soya-bean inhibitor added to a constant amount of trypsin, on the basis of this being a unimolecular reversible reaction. The curve in Fig. 5 is similar to that derived from Kunitz's equation though the agreement was not exact.

A comparison of the inhibitory power of various sera on trypsin can be made by reference to the curves obtained when various amounts of serum were added to a given amount of enzyme, such as crystalline trypsin (Fig. 6). A comparison of the volumes of each serum (measured on the curves) required to produce the same degree of inhibition shows good agreement when measured at $v = 500$ and $v = 400$ (Table 2). Since eight samples of normal human serum taken on two successive days from four persons showed little or no difference in inhibitory power when compared in this manner, fresh normal human serum was used as the basis of comparisons carried out at different periods during the course of the work. Similar lack of variations in inhibitory power within individual species was encountered in the case of other normal animal sera.

Table 2. *Comparison of inhibitory power of various sera against crystalline trypsin for values of v in Fig. 6*

| | $v = 500$ | | $v = 400$ | |
|---|---|---|---|---|
| Serum | Serum (ml.) | Volumes containing same activity relative to rabbit serum = 100 | Serum (ml.) | Volumes containing same activity relative to rabbit serum = 100 |
| Rabbit | 0·66 | 100·0 | 1·22 | 100·0 |
| Human | 0·42 | 63·5 | 0·72 | 59·0 |
| Horse | 0·35 | 53·0 | 0·60 | 49·0 |
| Sheep | 0·20 | 30·0 | 0·40 | 32·8 |
| Human (pregnancy) | 0·19 | 28·5 | 0·37 | 30·0 |
| Human (tuberculosis) | 0·13 | 19·9 | 0·25 | 20·5 |

### *Characteristics of the normal serum trypsin inhibitor*

*Stability of serum trypsin inhibitor.* The inhibitor was more unstable in the acid than in the alkaline range and there was some species variation (Table 3).

Table 3. *Stability of serum trypsin inhibitor under various conditions*

(In all experiments except the last, the serum was first diluted to 10% (v/v) in saline.)

| Time | Temp. (°) | pH | Loss in inhibitory power (%) Sheep | Horse |
|---|---|---|---|---|
| 2 hr. | 37 | 4·6 | 27 | 80 |
| 2 hr. | 37 | 9·6 | Nil | Nil |
| 30 min. | 60 | 7·4 | 20 | 46 |
| 30 min. | 70 | 7·4 | 33 | 70 |
| 30 min. | 80 | 7·4 | 95 | 70 |
| 12 months | 4 | — | Nil | Nil |

There was a considerable loss above 60° in both horse and sheep sera. The loss on storage at 4° in both sera was very small, and there was even evidence of some increase.

*Effect of fat solvents.* The most marked destruction of the inhibitor was obtained by shaking and incubation with chloroform which acts as a protein denaturant (Table 4). Several attempts were made to repeat the findings of Ungar (1945), who claims that, whilst after ether extraction both the aqueous and ether fractions of normal guinea-pig serum have slight antitryptic action, their antitryptic power on recombination is equal to that of the original serum. No details are given by Ungar, but in the present studies neither prolonged shaking nor reflux distillation for 20 min. of both guinea-pig and horse sera with ether removed more than a small fraction (often none) of the total activity, and this slight loss could not be restored by readdition of the extract. Ether extraction at −20° resulted in a 20% loss which was not restored by readdition of the ether soluble fraction. The inhibitor could not be extracted by acetone or chloroform.

Table 4. *Stability of sheep serum inhibitor on exposure to fat solvents*

(10% (v/v) solvent added, shaken 10 min. and incubated 5 hr. at 37°; activity measured at final serum dilution 1/1600 (v/v) against 100 units crystalline trypsin.)

| | v | Loss in inhibitory power (%) |
|---|---|---|
| Trypsin alone | 575 | — |
| Trypsin + serum treated with saline | 163 | — |
| " " " " acetone | 290 | 32 |
| " " " " ethanol | 188 | Nil |
| " " " " ether | 157 | Nil |
| " " " " chloroform | 476 | 75 |

*Precipitation by trichloroacetic acid and dialysis.* The antitryptic factor was precipitated with a loss of about 50% by the addition of an equal volume of 5% (w/v) trichloroacetic acid to sheep serum. The supernatant fluid was without activity. Sera diluted 1:10 and brought to various pH values were pressure-dialyzed through cellophan membranes, but the filtrates were without activity.

*Salt fractionation.* Serum treated with ammonium sulphate showed a marked loss in inhibitory power unless it was first buffered with sodium glycerophosphate, 7% (w/v). Under these conditions at least 90% of the total activity of sheep serum could be recovered (Table 5). A lower final yield was obtained on fractionation by 18% sodium sulphate for 1 hr. at 37° using horse serum. The inhibitor was present in both albumin and globulin fractions although there was a marked reduction in the inhibitory power of either fraction on subsequent precipitation. Separated electrophoretically both fractions were inhibitory, the globulin being about twice as active on a weight basis.

Table 5. *Comparison of inhibitory activity of serum fractions against crystalline trypsin after salting out*

| Fraction ... | Inhibitory activity as % of original serum: Albumin | Globulin | Total, reconstituted |
|---|---|---|---|
| Sheep serum once fractionated with $(NH_4)_2SO_4$ | 65 | 20 | 80 |
| Horse serum once fractionated with $Na_2SO_4$ | 32 | 13 | 65 |

A similar fractionation to that made by Beloff (1946) with ammonium sulphate showed that about two thirds of the total inhibitory activity of the albumin fraction lay in the more soluble part (Table 6), that is the fraction precipitated by full saturation with ammonium sulphate at pH 4·2, and that on a weight basis this was about six times as active as either the less soluble fraction precipitated at pH 4·2 and 50% (w/v) saturation or the globulin fraction. A further precipitation of the more soluble fraction corresponding to Beloff's $C_1$ showed a loss of 50% on a weight basis. The figures given are very approximate, but serve to illustrate the wide difference in inhibitory power of the different fractions.

Table 6. *Comparison of inhibitory activity of various sheep serum protein fractions following ammonium sulphate precipitation*

| | Globulin | Total albumin (fraction *A*) | Albumin precipitated by 50% saturation with $(NH_4)_2SO_4$ at pH 4·2 (fraction *B*) | Albumin precipitated by 100% saturation with $(NH_4)_2SO_4$ at pH 4·2 (fraction $C_1$) |
|---|---|---|---|---|
| Activity recovered expressed as percentage total activity of serum | 20 | 65 | 12 | 45 |
| Concentration of fraction in serum (%) | 3·0 | 3·5 | 1·92 | 1·17 |
| $\frac{\text{Activity}}{\text{Concentration}}$ | 6·6 | 18·5 | 6 | 38 |

*Serum antitrypsin factor of Schmitz.* Schmitz (1937, 1938) claimed to have separated an antitryptic factor from serum, which appeared to resemble a polypeptide, and which he believed served as a bound inactivator of the plasma trypsin. The antitryptic factor was obtained in acetone extracts of human plasma and by fractional precipitation of ox blood with ammonium sulphate following the addition of 2 vol. of 0·5 N-sulphuric acid. In the present study no inhibitory activity was found in acetone extracts of human plasma following Schmitz's directions. The fractional precipitation of 1·5 l. of ox blood gave approximately 180 mg. of material, representing a total inhibitory activity of only some 0·02% of the original 1·5 l. Two thirds of the product was ammonium sulphate, and this cannot be removed according to Schmitz since the inhibitor itself is dialyzable. A similar product was obtained from human blood which was not destroyed by the addition of streptokinase as might have been expected from its supposed nature.

*Variation in antitryptic level*

(*a*) *Normal animals.* Although the antitrypsin levels of different animal species varied considerably there was little or no variation between individual members of the same species.

(*b*) *Anaphylaxis.* Burdon (1942) claimed that there was a 100% increase in the antitryptic power of the sera of guinea pigs sensitized with egg albumin which fell rapidly to normal following injection of a shocking dose. None of these changes could be confirmed.

(*c*) *Immunity.* Grob (1943) claimed that the daily intramuscular injection of crude trypsin in rabbits over 4 weeks resulted in a rise of between 70 and 250% in the antitrypsin titre of the animals, and that a similar, though less constant, rise was obtained in 4–8 days by the daily oral administration of the same product. In the present studies there was a rise of some 15% in the antitrypsin activity of the serum of a rabbit injected daily subcutaneously for 4 weeks with crude trypsin, but no

rise was observed following oral administration as claimed by Grob.

(*d*) *Alterations in disease.* Many observers have noted an altered level of serum antitrypsin activity in various pathological conditions in man and animals, usually associated with a considerable degree of cachexia. The sera of seven persons all showing marked cachexia were examined. An increase of about 150 % was found in the antitryptic titre of the serum of three cases of tuberculosis. The remaining four (two tubercular and two non-tubercular) were normal.

(*e*) *Pregnancy.* The sera of eight women all in the terminal stages of pregnancy showed a uniform increase in the antitrypsin level of about 100 %.

## DISCUSSION

The exact nature of the serum factor responsible for trypsin inhibition is still in doubt. Although associated with all fractions obtained by fractional salting out, it may yet be a single factor which is adsorbed on other proteins during precipitation. All attempts to separate it from albumin either by solvent extraction, adsorption or dialysis, under various conditions, have been unsuccessful, thus confirming the findings of other authors. Recently, Lineweaver & Murray (1947) have shown that the trypsin-inhibitory property of egg white is due to ovomucoid, and it is possible that the antitryptic action of serum is associated with a similar small molecular compound such as serum mucoid or seroglycoid (Hewitt, 1937). Unfortunately, the preparation of either of these substances, if they are indeed distinct (Meyer, 1938; Rimington, 1940), is carried out by methods likely to impair antitryptic properties, such as successive fractionation and crystallization at the isoelectric point (Hewitt, 1937), or by removal of the other proteins by boiling (Rimington, 1940). It will, therefore, be necessary to devise other methods for their isolation if this matter is to be tested.

A comparison of these studies with those of Beloff (1946) makes it clear that the serum inhibitor of skin protease and serum antitrypsin are identical. Serum trypsin is likewise inhibited by the same factor (Christensen, 1946). Beloff failed, however, to find any inhibitory activity against skin protease in the globulin fraction of serum, as did other authors who used pancreatic trypsin. In the present studies inhibitory activity was present in the globulin after two precipitations with ammonium or sodium sulphate, although it was much less than the activity after one precipitation. Globulin separated electrophoretically was about half as inhibitory on a weight basis as was albumin when measured against crystalline trypsin.

Finally there is no evidence that the inhibitor or portion of the inhibitor can be extracted with ether as claimed by Ungar (1945), nor is there any real evidence that serum contains a polypeptide inhibitor similar in its properties to the pancreatic trypsin inhibitor isolated by Kunitz & Northrop (1936), as is claimed by Schmitz (1937, 1938). The treatment of whole blood with 0·5 N-sulphuric acid followed by fractionation between 40 and 70 % saturation with ammonium sulphate undoubtedly gives a product with weak inhibitory powers. The hypothesis that this product is a specific inactivator for plasma trypsin, similar to Northrop's inhibitor for pancreatic trypsin, is very much open to doubt, since it rests on nothing more than a comparison of the stoicheiometric properties of the two substances. Schmitz was unable to free his inhibitor from considerable amounts of ammonium sulphate, and his estimate of enzyme inhibition was based on the increase in non-protein nitrogen following relatively short hydrolysis of casein by trypsin. If the substance obtained by this method from human plasma is the specific inactivator for plasma trypsin, it would be expected to lose activity in the presence of streptokinase, which was not the case. From the method of fractionation employed it might reasonably be concluded that the substance is part of the serum albumin which has escaped precipitation with the blood, but is partly denatured.

## SUMMARY

1. Trypsin and trypsin inhibitors can be assayed viscosimetrically with gelatin as a substrate, using the flow-time index relationship developed by Swyer & Emmens (1947) for the assay of hyaluronidase. Some of the principles involved are discussed.

2. The serum trypsin inhibitor is relatively stable and following fractional precipitation or electrophoresis it is associated with all fractions though mainly with the most soluble albumin fraction. It cannot be removed or destroyed by ether extraction, but is easily damaged by chloroform. It is sensitive to increased hydrogen-ion concentration and is lost slowly, if at all, on storage.

3. A small amount of inhibitory material was obtained from human and sheep blood using the method described by Schmitz (1938). The human material was not affected by the addition of streptokinase and it is doubtful if it has the role assigned to it by this author.

4. There is a marked species variation between the antitryptic power of different animal sera, but little variation between the sera of individual members of the same species.

5. No increase was found in the antitrypsin level of rabbit sera following feeding of trypsin and a slight increase after repeated daily injections of trypsin. No change was observed in the inhibitory power of guinea pig sera following protein sensitization either before or after the production of anaphylaxis.

6. The sera of certain persons suffering from tuberculosis showed a marked increase in inhibitory power as did the sera of all women examined in the terminal stages of pregnancy.

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# Protease Inhibitors

## 2. BACTERIAL PROTEASES AND THEIR INHIBITORS

By E. S. DUTHIE and L. LORENZ, *Lister Institute, Elstree, Herts*

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Inhibition of bacterial proteases by normal or immune sera has been studied by various authors, although the results are not always in agreement, probably because of the failure to recognize that the filtrates might contain several proteases, only one of which was inhibited by normal serum. In addition, sensitivity to inhibitor might vary according to the organism and type of culture used. von Dungern (1898) found that the hydrolysis of gelatin by filtrates of *Bacillus anthracis*, *Vibrio cholerae* and *Staphylococcus aureus* was inhibited by immune but not by normal serum. Bertiau (1914) found that normal human serum had no inhibitory action on the proteases of *Bacillus subtilis* and *Pseudomonas pyocyanea*, although specific immune serum inhibited. Blanc & Pozerski (1920) showed that gelatin hydrolysis by filtrates of *Clostridium sporogenes* was inhibited by normal horse serum, which had no action on filtrates of *Cl. histolyticum*. Pozerski & Guelin (1938) showed that raw egg white had similar properties with regard to these filtrates. A more comprehensive study by Maschmann (1937*a*, *b*, 1938*a*, *b*, *c*, *d*, *e*) showed that at least three extracellular proteases could be recognized, and that a bacterial filtrate might contain one or more of these depending on the organism and method of preparation. A summary of Maschmann's findings is given in Table 1.

Smith & Lindsley (1939), in a study of the hydrolysis of gelatin by filtrates of various Clostridia, showed that, whilst normal serum inhibits proteases of non-pathogenic bacteria, it has no action on those of pathogens such as *Cl. histolyticum*, *Cl. welchii* and *Cl. oedematiens*. Filtrates of a rough strain of *Cl. histolyticum* were, however, inhibited by rabbit serum, but experiments showed that,

Table 1. *Classification of bacterial proteases according to Maschmann* (1937*a*, *b*, 1938*a*, *b*, *c*, *d*, *e*)

| Protease | Organism | Substrate | Activator | Inhibition by normal serum |
|---|---|---|---|---|
| (1) 'Pyocyaneus protease' | *Ps. pyocyanea** <br> *Chromobact. prodigiosum** <br> *Ps. fluorescens liquefaciens** <br> *B. mesentericus** | All proteins and peptones | Not required | + |
| (2) 'Gelatinase' | *Cl. welchii* <br> *Cl. septicum* <br> *Cl. histolyticum* <br> *Cl. chauvei* | Gelatin only | Not required | – |
| (3) 'Anaerobiase' | *Cl. welchii* <br> *Cl. septicum* <br> *Cl. histolyticum* <br> *Cl. botulinum* | All proteins | Requires low redox potential (SH compounds best) | – |

* These organisms are aerobes: all others listed above are anaerobes.

whilst the inhibitory power of serum against the filtrates of a rough strain of *Cl. histolyticum* was lost on storage or on treatment with ammonium sulphate to half saturation, there was no corresponding loss when tested against trypsin. Electrophoretic separation of normal rabbit serum showed that the inhibitory power against trypsin lay entirely in the albumin fraction, whilst that against the protease produced by a rough strain of *Cl. histolyticum* was present also to some extent in the $\beta$-globulin fraction.

Some explanation of these findings can be found in the studies of Maschmann (1937*a*, *b*, 1938*a*, *b*, *c*, *d*, *e*), who showed that the pathogenic Clostridia, *Cl. welchii*, *Cl. histolyticum*, produce a specific gelatinase not inhibited by serum. Bidwell & van Heyningen (1948) have since confirmed the substrate specificity of the *Cl. welchii* gelatinase.

## EXPERIMENTAL

*Culture filtrates.* The cultures of *Staph. aureus*, *Chromobacterium prodigiosum* and *Ps. pyocyanea* were grown 18–24 hr. in digest broth continuously shaken. Those of *B. anthracis* and *V. cholerae* were grown in Roux bottles on agar for 18–24 hr. and washed off in saline. *Cl. bifermentans* was grown on the medium of Macfarlane & Knight (1941). *Cl. welchii* Types A and D, and *Cl. histolyticum* were grown 8–18 hr. in a meat infusion broth containing 0·6% (w/v) glucose and 0·001 M-thioglycollic acid. The *Cl. welchii* Type B culture was grown in a glucose peptone broth containing thioglycollic acid and sodium glycerophosphate. All cultures with the exception of those grown on agar were filtered free of organisms; those grown in liquid media were concentrated 5–30 times by pressure dialysis, and all were finally dialyzed against saline and stored under toluene at 4° or frozen.

*Estimation of enzyme and inhibitor activity.* The majority of estimations were made by the viscosimetric technique already described (Duthie & Lorenz, 1949). A standard preparation of crystalline trypsin which was assigned the arbitrary value of 20,000 units/mg. was the standard of comparison. The casein hydrolysis experiments described in Table 2 had the enzyme concentration given in the second column of the table in a total of 1 ml. of 1% casein, and were incubated 3 hr. at 37°, after which the mixtures were precipitated with 2 vol. of 5% (w/v) trichloroacetic acid. The increase in acid-soluble 'tyrosine' against control mixtures not incubated was estimated by the Folin and Ciocalteu phenol reagent.

All inhibitors were incubated for 10 min. at 37° with the enzyme before the addition of substrate. Milk clotting times were estimated by adding equal volumes of fresh milk to the enzyme concentration given. Collagenase activity was measured as described by Oakley, Warrack & van Heyningen (1946).

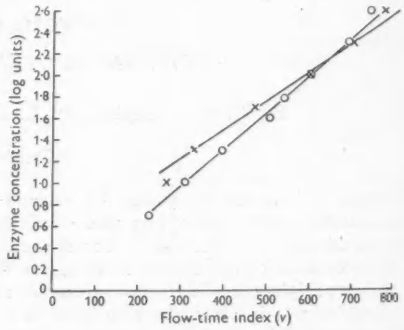


Fig. 1. Relationship between flow-time index ($v$) and logarithm of enzyme concentration for two bacterial filtrates: ×, *Ps. pyocyanea*; o, *Staph. aureus* (100 units of bacterial filtrate $\equiv 5\,\mu$g. of crystalline trypsin, i.e. a flow-time index of approximately 600).

## RESULTS

### *Measurement of proteolytic activity*

*Effect of bacterial proteases on gelatin.* The examination of a number of bacterial proteases (Fig. 1) showed that the flow-time index ($v$) plotted against

the logarithm of enzyme concentration gave a straight line over at least part of the range $v = 600$–200. The slope of the line in the case of certain organisms approximates to that for trypsin, lying between 1/380 and 1/450 (Duthie & Lorenz, 1949), but in the case of other organisms, except *Cl. welchii* Type B, it lay between 1/250 and 1/300

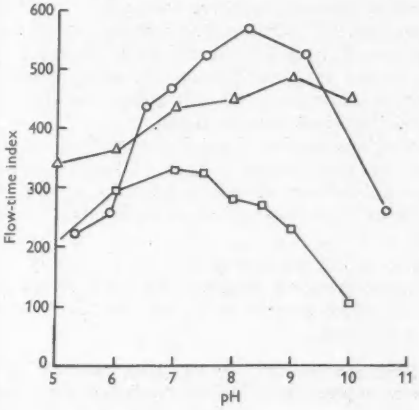


Fig. 2. Effect of pH on enzyme activity of filtrates of aerobic bacteria as measured by the flow-time index. △, *Chromobact. prodigiosum*; □, *Staph. aureus*; ○, *V. cholerae*.

(Fig. 1, Table 2). Thus dilution of these latter decreased the rate of gelatin hydrolysis much less than in the case of trypsin. Quantitative viscosimetric comparisons of protease activity between these two types of filtrate were, therefore, impossible, and all solutions used in Table 2 were standardized so as to have a $v$ value of about 600 after 20 min. hydrolysis of gelatin.

*Effect of* pH. The filtrates chosen for comparison were selected from those bacteria which showed well-marked proteolytic activity in crude unconcentrated filtrates. The majority of such filtrates showed optimal activity between pH 7 and 9 (Figs. 2, 3), although a filtrate of *Cl. welchii* Type A

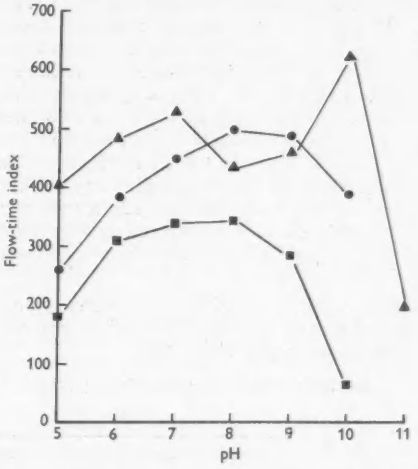


Fig. 3. Effect of pH on enzyme activity of filtrates of anaerobic bacteria as measured by the flow-time index. ▲, *Cl. welchii* Type A filtrate; ■, *Cl. welchii* Type B filtrate; ●, *Cl. histolyticum*.

showed two optima, one at pH 7 and the other at pH 10, indicating the presence of two enzymes. Walbum & Reymann (1934) obtained similar curves for this organism, although their optima were slightly lower. All filtrates showed good activity around pH 7·6, which was that used in the majority of experiments.

Table 2. *Effect of the same concentration of different bacterial filtrates on different substrates and a comparison of the inhibitory activity of normal sheep serum*

| Organism | Gelatinase activity (flow-time index) | | Slope of gelatin activity curve | Activity against casein measured as μg. 'tyrosine' liberated/ml. digest | | Milk clotting time (min.) |
|---|---|---|---|---|---|---|
| | Enzyme alone | Enzyme and 10% (v/v) sheep serum | | Enzyme alone | Enzyme and 10% (v/v) sheep serum | |
| *Staph. aureus* (113344) | 612 | 406 | 1/270 | 102 | 48 | — |
| *B. anthracis* | 603 | 0 | 1/250 | 110 | 0 | 18 |
| *Chromobact. prodigiosum* | 646 | 0 | 1/440 | 118 | 18 | 15 |
| *Ps. pyocyanea* | 620 | 0 | 1/380 | 111 | 0 | 30 |
| *B. megatherium* | 598 | 0 | 1/260 | 100 | 0 | 3 |
| *Cl. welchii* Type A | 600 | 550 | 1/450 | 0 | 0 | — |
| *Cl. welchii* Type B | 570 | 0 | 1/110 | 80 | 39 | 3 |
| *Cl. bifermentans* | 613 | 0 | 1/280 | 84 | 0 | 4 |
| *Cl. histolyticum* | 645 | 325 | 1/410 | 66 | 0 | — |
| Crystalline trypsin | 606 | 0 | 1/400 | 81 | 0 | — |

### *Serum inhibition*

*Action of serum.* The addition of serum to a final concentration of 10% (v/v) affected the hydrolysis of both gelatin and casein in the case of all the bacterial filtrates studied (Table 2). Complete inhibition of gelatin hydrolysis was found in the case of four of the five filtrates of aerobic bacteria and in two of the anaerobic bacteria. Wide variation in sensitivity to serum was encountered among those completely inhibited by 10% sheep serum; a 50% inhibition was produced in 100 units of a filtrate of *Chromobact. prodigiosum* by a final dilution of 1·25% serum, whereas the same effect was produced in 100 units of a filtrate of *Ps. pyocyanea* by as little as 0·05% serum. The inhibitory power of different animal sera varied according to the species (Table 3), sheep being very much more inhibitory as

Table 3. *Comparison of inhibitory effect of normal animal sera on a culture filtrate of* Ps. pyocyanea *as measured by flow-time index*

(Filtrate 100 units/ml.: sera 0·16% (v/v).)

| | Sera | | | |
|---|---|---|---|---|
| | Rabbit | Human | Horse | Sheep |
| Flow-time index | 385 | 390 | 330 | 190 |

in the case of crystalline trypsin (Duthie & Lorenz, 1949). The interaction between serum and a filtrate of *Ps. pyocyanea* was immediate, there being no increase in inhibition if the mixture was incubated for varying periods prior to the addition of gelatin.

*Effect of age and heating on serum.* Specimens of horse and human sera kept at 4° rapidly lost part of their inhibitory power against bacterial filtrates, though this was not so noticeable in the case of sheep serum. Table 4 shows that, in a comparison

Table 4. *Comparison of loss in inhibitory effect of stored normal sera on a bacterial filtrate* (Ps. pyocyanea) *and on crystalline trypsin*

(Sera stored 1 year at 4°. Enzyme 100 units/ml.)

| | Loss in inhibitory power (%) | |
|---|---|---|
| | Human serum | Sheep serum |
| Filtrate of *Ps. pyocyanea* | 66 | 80 |
| Crystalline trypsin | 30 | Nil |

of fresh and stored human and sheep sera, the loss in inhibitory activity is very much greater when measured against a bacterial filtrate than against crystalline trypsin. The comparison is based on the assumption that the stored sera had originally the same inhibitory power as the fresh sera used. Even if this were not the case, the figures show clearly that inhibitory activity against trypsin and a bacterial filtrate are unrelated, since the fresh sheep serum was equal in inhibitory power to the old serum when measured against crystalline trypsin, though five times as active when measured against the bacterial filtrate. The inhibitor against bacterial filtrates was very sensitive to age and to acidity, more than 90% of the activity of sheep serum being lost after 2 hr. at 37° and pH 4·6 or 30 min. at 70° in neutral solution. The loss in activity against trypsin under these conditions was less than one third (Duthie & Lorenz, 1949).

*Salt fractionation.* Precipitation with ammonium sulphate provided further proof (Table 5) that the serum inhibitor against a bacterial filtrate was different from that against crystalline trypsin since

Table 5. *Comparison of inhibitory power of sheep serum fractions measured against* 100 *units of crystalline trypsin and a culture filtrate of* Ps. pyocyanea

(Fractions separated by $(NH_4)_2SO_4$ and activity expressed as percentage of inhibitory activity of whole serum.)

| | Globulin | Albumin precipitated by complete saturation with $(NH_4)_2SO_4$ at pH 4·2 (Fraction $C_1$) |
|---|---|---|
| Crystalline trypsin | 20 | 45 |
| *Ps. pyocyanea* | 38 | 2·8 |

the more soluble albumin fraction $C_1$, precipitated by full saturation with ammonium sulphate at the isoelectric point, contained nearly half the original activity against trypsin but only 3% of the original activity when measured against the bacterial filtrate. Similar results were obtained with fractions separated electrophoretically. Thus while both globulin and albumin fractions are inhibitory, though in different degree, to crystalline trypsin, the bulk of the inhibitory action against bacterial proteases is associated with the globulin.

### *Effect of other inhibitors*

*Egg white and soya bean.* Neither soya-bean trypsin inhibitor nor pancreatic trypsin inhibitor had any action on the proteases of either *Staph. aureus*, *Ps. pyocyanea* or *Chromobact. prodigiosum*. Raw egg white resembled serum with regard to the varying sensitivity of different bacterial filtrates (Table 6). Ovomucoid purified according to the method of Lineweaver & Murray (1947) was active against 100 units of crystalline trypsin at 1:2,000,000, but was without activity against bacterial filtrates at 1:1000.

Table 6. *Comparison of sensitivity of different bacterial filtrates to serum and to egg white*

| | Concentration of inhibitor (%) | Inhibition of proteolysis (%) | | |
|---|---|---|---|---|
| | | *Ps. pyocyanea* | *Chromobact. prodigiosum* | *Cl. welchii* Type A |
| Human serum | 1·25 | 100 | 50 | Nil |
| Egg white | 2·5 | 62 | 33 | Nil |
| Soya bean | 0·1 | Nil | Nil | Nil |

*Specific antisera.* When specific antiserum is added to a constant amount of a culture filtrate of *Staph. aureus* or of *Cl. welchii* Type A, and the flow-time index is plotted against the units of antiserum added, the curves obtained are not dissimilar from those obtained by the addition of normal serum to either crystalline trypsin (Duthie & Lorenz, 1949) or to filtrates of bacteria (Fig. 4). The viscosimetric

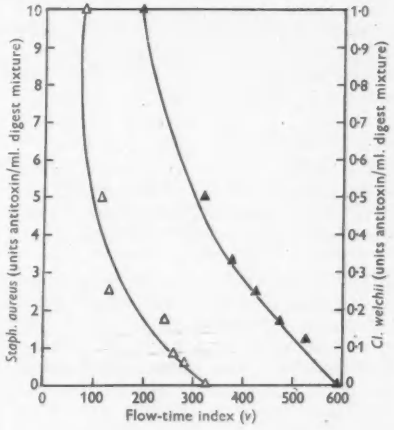


Fig. 4. Effect of increasing amounts of specific antiserum on filtrates of *Staph. aureus* and *Cl. welchii* Type A. (Staphylococcal antiserum was a globulin fraction, *Cl. welchii* antiserum was a pepsin-digested globulin.) △, *Staph. aureus*; ▲, *Cl. welchii*.

method can thus be used in the estimation of the antiprotease component of any antiserum. Both staphylococcal and *Cl. welchii* Type A antisera were specific in their antiprotease activity and the *Cl. welchii* Type A antiserum did not neutralize the proteases in *Cl. welchii* Type B filtrates.

### *Comparison of filtrate activities*

Table 2 shows that for all five filtrates of aerobic bacteria examined there was a very good correlation between casein and gelatin hydrolysis, comparing the $v$ value and acid-soluble 'tyrosine' liberated, indicating that the same enzyme is probably involved. The filtrates from Clostridia showed a much greater degree of variation and, in addition, a lower ratio of casein to gelatin hydrolysis. Of these four filtrates, only *Cl. welchii* Type A failed to hydrolyze casein, and to hydrolyze gelatin in the presence of serum. The hydrolysis of gelatin by a filtrate of *Cl. histolyticum* was only partly inhibited by serum, and both filtrates presumably contain a specific gelatinase. These two filtrates attacked native collagen in the form of muscle and tendon, but collagenase and gelatinase properties are not due to the same enzyme, since two purified preparations of collagenase received from Miss E. Bidwell had lost completely their power of attacking native collagen following 4 months' storage at 4°, while retaining their gelatinase properties unimpaired.

None of the filtrates in Table 2 showed increased proteolysis in the presence of thioglycollic acid. Only certain filtrates clotted milk, and then the rate of reaction was not related to protease activity as measured either on gelatin or casein. The clotting of milk was inhibited by normal sera and especially by the globulin fraction. The albumin fraction used in the experiment of Table 5 had little or no inhibitory effect.

## DISCUSSION

In contrast to serum antitrypsin, which is associated with the more soluble albumin fraction, the serum inhibitor of bacterial proteases is associated mainly with the globulins. This property is also much more labile than in the case of serum antitrypsin, but it does not disappear completely on storage as stated by Smith & Lindsley (1939). Likewise, whilst considerable differences exist between the inhibitory power of the sera of different species, it is never absent as claimed by Maschmann (1937*b*), and such discrepancies as have been reported are due either to varying sensitivity of the proteases tested or to failure to use fresh serum, since the inhibitory titre of horse serum can fall very rapidly even at 4°. The rapid combination of the serum inhibitor of bacterial proteases with enzyme resembles that of an antigen-antibody reaction, and further distinguishes this inhibitor from serum antitrypsin. Todd (1947) has found that the serum of about 5% of normal horses and 25% of normal humans investigated contained

an inhibitor for a streptococcal protease which he studied. The inhibitor also lay in the globulin fraction and suggested some form of 'natural antibody'. Wide variations in antiprotease titre, such as were found by Todd, were not noticed in the present studies, but sufficient work has not been done on this point. All human and animal sera investigated inhibited susceptible bacterial proteases, and the inhibition would, therefore, appear to be part of a non-specific immunity reaction.

Recently Haas (1946) has studied the inhibitory action of serum on hyaluronidase both of testicular and of bacterial origin. Haas finds that, like the reaction between serum antitrypsin and trypsin, the amount of inhibition increases with time of contact of enzyme and serum prior to incubation with substrate. Similarly, the inhibitor is destroyed by short treatment with acid and by heating to 53° for 15 min. It is apparently assumed that the same substance is responsible for the inhibition of both testicular and bacterial hyaluronidase, since the source of the enzyme used in these experiments is not specified. On the basis of these findings, Haas concludes that the serum inhibitor of hyaluronidase is an enzyme which he terms antivasin 1. Since the inhibitory properties of serum for both trypsin and bacterial proteases are analogous to and closely resemble those antihyaluronidase properties described by Haas, one might equally regard them as being due to an enzyme. While such a possibility might be considered, it is felt that the evidence produced by Haas is insufficient.

The filtrates of *Cl. welchii* Type A and *Cl. histolyticum* clearly contain the substrate-specific gelatinase described by Maschmann (1937*a*, *b*, 1938*b*), which is only partly inhibited by very large amounts of normal serum such as 10% (v/v) used in Table 2. Although gelatinase is closely associated with collagenase in the purification process used by Bidwell & van Heyningen (1948), the two are not identical, since collagenase activity alone is lost on storage or on dialysis at pH 10·0 (Bidwell, personal communication).

## SUMMARY

1. The flow-time index relationship described by Swyer & Emmens (1947) can be used for the viscosimetric assay of bacterial proteases and their inhibitors.

2. The serum inhibitor active against bacterial proteases is a labile component of the globulin fraction, in contrast to the relatively more stable serum antitrypsin, which is found mainly in the albumin fraction. The serum of different animal species varies in its inhibitory power.

3. Bacterial proteases vary in their sensitivity to the serum inhibitor, the specific gelatinase found in certain Clostridia being relatively insensitive; filtrates sensitive to serum are also inhibited by egg white, but all are unaffected by ovomucoid, soya bean or by pancreatic trypsin inhibitors.

4. *Cl. welchii* Type A gelatinase is inhibited by specific antiserum, and the potency of different antisera can be compared viscosimetrically. The gelatinase activity is independent of collagenase activity.

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# The Measurement of Radioactive Sulphur ($^{35}S$) in Biological Material

By L. YOUNG,* M. EDSON and J. A. McCARTER
*Department of Biochemistry, University of Toronto*

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The only known radioactive isotope of sulphur which is suitable for biochemical investigations is $^{35}S$. During the period 1941–5, $^{35}S$ was used in this laboratory as a tracer isotope in biochemical studies of mustard gas (di-2-chloroethyl sulphide), certain mustard gas derivatives, and 2:3-dimercaptopropanol (BAL). These investigations were made possible as a result of $^{35}S$ being made available for the work from sources in the U.S.A., and through the kindness of Dr F. C. Henriques, jun. of Harvard University, who supplied the radioactive material in the form of mustard gas. It was during the course of the work with radioactive mustard gas that the present method of measuring $^{35}S$ in biological material was developed.

$^{35}S$ has a half-life period of 87·1 days (Hendricks, Bryner, Thomas & Ivie, 1943) and it emits $\beta$ particles with a maximum energy of 0·120 MeV (Kamen, 1941). The low-energy $\beta$ particles are easily stopped by relatively thin layers of absorbing material, and in order to measure $^{35}S$ accurately it is necessary to make corrections for the absorption of the radiation by the radioactive material itself. Correction for self-absorption of the radiation is facilitated by the separation of the isotope as a constituent of a sulphur compound. The radioactivity of this compound is then determined in an apparatus sufficiently sensitive to measure soft radiation. Such a procedure can be developed by modifying a gravimetric method for the determination of sulphur in such a way that the final precipitate is collected in a form suitable for the measurement of its radioactivity. A relation can then be obtained experimentally between the thickness of the precipitate in this form and its radioactivity.

Several methods have been described in which the material containing $^{35}S$ is oxidized and the sulphate is precipitated as the barium or benzidine salt, the radioactivity of which is measured. Among the oxidation procedures which have been used are the Pirie (1932) method (Tarver & Schmidt, 1939; Boursnell, Francis & Wormall, 1946), the Carius method (Henriques, Kistiakowsky, Margnetti & Schneider, 1946), oxidation with concentrated nitric acid and hydrogen peroxide (Seligman & Fine, 1943), and oxidation with sodium carbonate and sodium peroxide (Dziewiatkowski, 1945).

* Now at St Thomas's Hospital Medical School, London, S.E. 1.

The first method used in this laboratory (Young, 1941) for the separation of sulphur in biological material for radioactivity measurements was based on the Masters (1939) modification of the ter Meulen (1922) procedure for determining sulphur. The type of apparatus required and the amount of attention needed for the conduct of this method rendered it unsuitable for the performance of the large number of analyses which had to be carried out and it was eventually abandoned. Various modifications of the nitric acid-perchloric acid oxidation procedure (Pirie, 1932; Masters, 1939) were also tested, but in our hands they yielded low results when used for the determination of the sulphur content of some of the compounds being studied (e.g. mustard gas, thiodiglycol).

The procedure finally adopted (Patrick & Young, 1943; McCarter & Young, 1944) was based on that originally described for the analysis of organic compounds by Carius (1860). A study was made of the conditions which must be observed if this method is to be applied to biological material. After the material had been oxidized, the sulphate was precipitated as benzidine sulphate by a procedure based on that described by Niederl, Baum, McCoy & Kuck (1940) for the microdetermination of sulphur in organic compounds. The benzidine sulphate precipitate was collected by a method designed to reduce, as far as possible, self-absorption of the radiation by the compound during the measurement of its radioactivity. In order to correct for the small amount of self-absorption which did occur, the deposit of benzidine sulphate was weighed, and all radioactivity measurements were referred to the same weight of benzidine sulphate. The decision to separate the sulphate as the benzidine salt rather than as barium sulphate was based mainly on the finding that benzidine sulphate yields a uniform deposit suitable for radioactivity measurements much more readily than does barium sulphate.

The radiation from $^{35}S$ can be measured in several different ways. Henriques *et al.* (1946) compared the Lauritsen quartz fibre electroscope and a counter with a Geiger tube with a very thin window, and found them to be equally satisfactory on the basis of accuracy and sensitivity, although these workers indicated a preference for the electroscope for the routine measurement of $^{35}S$. The apparatus used in the present work was an ionization chamber with

a direct current amplification system employing an FP-54 tetrode valve. This valve has been described by Metcalf & Thompson (1930). When it is operated in conjunction with a galvanometer, currents of the order of $10^{-14}$ amp. can be measured with comparative ease, and currents as small as $5 \times 10^{-18}$ amp. can be detected (DuBridge, 1931).

## EXPERIMENTAL

### *Conversion of the sulphur of organic compounds and biological material to benzidine sulphate*

Experiment showed that 1–2 mg. S yielded an amount of benzidine sulphate (8·8–17·6 mg.) suitable for radioactivity measurements, and it was necessary to oxidize an amount of material which contained this weight of S if the addition of non-radioactive sulphate to the sample was to be avoided. In order to do this it was necessary to know the approximate S content of the material being analyzed. There is little information on the total S content of tissues to be found in the literature. Kambayashi (1929) reported mean values of 2·22 and 2·42 mg., respectively, for the S content/g. of fresh liver and muscle of rats (body wt., 180–250 g.), and similar values for the tissues of other animal species have been reported (Silberstein, 1934; Brown & Klauder, 1935; Masters & McCance, 1939; Sherman, 1941). It appeared probable, therefore, that it would be necessary to develop an oxidation procedure which could be applied to 0·3–0·5 g. of fresh tissue. This was confirmed by experience.

Experiments were conducted in which the size of the Carius bomb, the amount of $HNO_3$, and the time and temperature of heating the bomb were varied until a satisfactory set of conditions was found for the oxidation of 0·3–0·5 g. of tissue. The methods used for the evaporation of the contents of the bomb and for the precipitation of the sulphate with benzidine hydrochloride were based on those used by Niederl *et al.* (1940) for the microdetermination of S in organic compounds. Benzidine sulphate separates well when centrifuged, and when $^{35}S$ was to be measured, the benzidine sulphate was separated and washed in a centrifuge tube before depositing it on a Petri dish for radioactivity measurements. When the method was applied to the gravimetric determination of S, the benzidine sulphate was collected on a microfilter with a fritted disk, washed, dried and weighed as described by Niederl *et al.* (1940). A detailed account of the procedure is given below.

*Procedure.* The following reagents were used. Red fuming $HNO_3$ (Baker's C.P. Analyzed. Sp.gr. 1·59–1·60).

NaBr. Analytical reagent grade.

Benzidine hydrochloride reagent. To 5 g. of pure benzidine hydrochloride were added 40 ml. of N-HCl and enough 50% (v/v) aqueous ethanol to give 250 ml. The solution was heated until it was just boiling. It was then cooled, filtered and stored in a dark glass-stoppered bottle. The solution was filtered from time to time.

Ethanol, 50 and 95% (v/v). These reagents were prepared by diluting pure ethanol with water, and were tested to ensure that they did not yield a significant amount of residue when evaporated to dryness.

Great care was taken in the cleaning of all glassware used in the measurement of $^{35}S$. This was especially important in the case of glassware which had been used in the analysis of strongly radioactive material.

The general procedure adopted for preparing and sealing the Carius bombs was that described by Niederl *et al.* (1940). The bombs were made from Pyrex glass tubing with an internal diameter of 2·2 cm. and an external diameter of 2·5 cm. The length of a bomb was based on the quantity and nature of the material to be oxidized, and in general the length was between 30 and 50 cm. Whenever possible, the amount of material oxidized was such that its S content was 1–2 mg. When it appeared probable that a sample of material which was being analyzed for its $^{35}S$ content contained less than 1 mg. S, sufficient non-radioactive $Na_2SO_4$ was added to raise the S content of the benzidine sulphate precipitate to at least 1 mg. The sample was introduced into the bomb together with 5–10 mg. of NaBr and 1·2 ml. of red fuming $HNO_3$ (in some analyses it was necessary to use larger amounts of $HNO_3$, and under these conditions bombs of greater length than 50 cm. were used). The open end of the bomb was drawn out to a thick-walled capillary and sealed immediately after the introduction of the reagents. The bomb was then heated at 300° in a Carius furnace for not less than 4 hr. After the bomb had been allowed to cool in the furnace, the tapered portion was heated gently to drive into the main part of the bomb any liquid which had condensed in the capillary. In order to release the pressure inside the bomb the tip of the capillary was then heated with a small hot flame until an opening was forced by the compressed gases. A scratch mark was then made around the bomb just below the tapered portion, and the bomb was cracked around this mark by applying a hot glass rod to it. The tapered portion was separated from the main part of the bomb and washed with water. These washings, together with the contents of the bomb, were washed with water (total vol. 25 ml.) into an evaporation tube and evaporated to dryness.

The evaporation tube consisted of a Pyrex boiling tube (20 × 2·5 cm.) with a short glass side arm attached at right angles 4 cm. from the mouth. The evaporation tube was closed by a cork (a new cork was used for each analysis) carrying an inverted L-shaped inlet tube with a constriction at the lower end. A short length of rubber tubing with a screw clip was attached to the upper end of the inlet tube. The evaporation tube was clamped in a boiling water bath, the side arm connected through a trap to a water pump, and a current of air drawn through the tube. The flow of air was regulated by means of the screw clip on the inlet tube, and the position of the inlet tube was adjusted so that its lower end was just above the surface of the solution. After the solution had evaporated to dryness the glass inlet tube was washed with water (total vol. 3 ml.). These washings were collected in the evaporation tube and served to dissolve the residue. Two ml. of 95% (v/v) ethanol were then added with shaking, followed by 1 or 2 ml. of benzidine hydrochloride reagent (the larger amount of this reagent was used whenever it appeared probable that the sample contained more than 2 mg. S). The precipitate of benzidine sulphate was stirred and allowed to stand for 30 min. The contents of the evaporation tube were then transferred to a tapered 15 ml. centrifuge tube. The evaporation tube was washed with small portions of 95% ethanol (total vol. 3 ml.) and these were added to the contents of the centrifuge tube. The precipitate after centrifuging was suspended in 10 ml. of 95% ethanol and recentrifuged. The precipitate was then again suspended in 95% ethanol, and transferred to a weighed Petri dish, 9 cm. in diameter. The precipitate was evapor-

ated to dryness by holding the Petri dish over an electric heater. During the evaporation the dish was manipulated in such a way that an even deposit of benzidine sulphate was obtained, covering the bottom of the dish. After cooling the dish was placed overnight in a desiccator. It was weighed and was then covered with its lid until the radioactivity of the deposit was measured.

The following procedure was used when the $^{35}S$ content of the analyzed sample was likely to exceed that which could be measured accurately with the ionization chamber apparatus. The aqueous solution obtained after dissolving the residue in the evaporation tube was made to a known volume. A measured portion of this solution was then diluted to 3 ml., non-radioactive $Na_2SO_4$ was added when necessary, and the addition of 95% ethanol and precipitation of the benzidine sulphate were carried out as described above.

Further details of the application of the method to the measurement of $^{35}S$ in organic compounds, tissues and excreta are given below.

*Analysis of organic compounds.* When the method was applied to a compound containing $^{35}S$, the sample (liquid or solid) was weighed in a small stoppered glass tube on a microbalance. The NaBr and red fuming $HNO_3$ were placed in the bomb, the stopper was removed from the tube containing the sample, the tube was introduced carefully into the bomb, and the bomb was sealed without delay.

*Analysis of tissues.* Rat tissues were placed in stoppered weighing bottles immediately after removal from the animal. A representative sample (0·3–0·5 g.) of each tissue was weighed, transferred to a bomb containing NaBr, red fuming $HNO_3$ was added, and the bomb was sealed.

Two procedures were used for the measurement of $^{35}S$ in blood. When the concentration of $^{35}S$ in the blood was such that accurate results could be obtained by the analysis of 0·2 ml. samples of blood, this amount was introduced into a bomb together with 1 mg. S (as non-radioactive $Na_2SO_4$), and the analysis completed as described above. When it was necessary to analyze larger samples, 1 ml. of blood, 1 ml. of water and 1 ml. of red fuming $HNO_3$ were placed in a bomb. The bomb was left unsealed and its contents were evaporated to small volume on an air bath. NaBr and 1 ml. of red fuming $HNO_3$ were then added, the bomb was sealed, and the analysis then completed in the usual way.

When the whole carcass of a rat was analyzed, it was placed in a beaker (2 l.) and red fuming $HNO_3$ (approx. 0·67 ml./g. of carcass) was added. The mixture was stirred from time to time and all of the carcass, with the exception of some fatty material, dissolved in the $HNO_3$ in a few hours at room temperature. The mixture was then transferred to a separating funnel and the $HNO_3$ solution was separated from the fatty material. The volume of the $HNO_3$ solution was measured, 0·5 ml. was transferred to a bomb which contained NaBr and 1 ml. of red fuming $HNO_3$ and the analysis completed as described. The fatty material was stirred vigorously with red fuming $HNO_3$ (about 50 ml. of acid were used for the fatty material from a rat weighing 150 g.), the volume of the suspension was measured, and a 1 ml. sample was sealed in a bomb which contained NaBr and 0·5 ml. of red fuming $HNO_3$.

*Analysis of excreta.* In order to determine the total $^{35}S$ content of urine, the volume of the urine was measured and 0·2 ml. was sealed in a bomb with 1 mg. S (as non-radioactive $Na_2SO_4$), NaBr and 1 ml. of red fuming $HNO_3$. For the analysis of faeces, the material was first ground in a mortar, and a weighed sample was then oxidized in the usual way.

*Gravimetric determination of sulphur.* The procedure differed from that already described in the following respects. The solution obtained by dissolving the residue in the evaporation tube was filtered to remove dust particles and any other insoluble material. The benzidine sulphate was precipitated under the conditions already described. It was then filtered on a microfilter with a fritted disk, washed with small portions of 50% (v/v) ethanol (total vol. 10 ml.), and dried to constant weight at 100–102°.

### *Measurement of the radioactivity of benzidine sulphate samples*

*Apparatus.* The apparatus consisted of an ionization chamber and an FP-54 electrometer circuit.* The circuit used was based on that described by DuBridge & Brown (1933). The FP-54 valve (General Electric Co., Schenectady, New York) was housed in an inverted position in an evacuated brass container directly above the ionization chamber. The ionization chamber was made of brass and was cylindrical in shape (internal dimensions: diameter, 11 cm.; height, 7 cm.). It consisted of an upper and a lower part which could be separated to permit the introduction of the Petri dish carrying the radioactive sample. The control grid of the valve was connected to the central electrode of the ionization chamber and a resistor of $10^{12}$ Ω., situated inside the housing of the valve, was connected between the control grid and earth. The sensitivity of the electrometer circuit was controlled by using an Ayrton shunt in conjunction with the galvanometer (no. 2500-g, Leeds and Northrup, Philadelphia, Pa.).

Current flow in the circuit was adjusted so that the galvanometer showed no deflexion when no radioactive material was present in the ionization chamber. When a radioactive sample was introduced into the chamber a galvanometer deflexion was produced which reached its equilibrium position in 10–15 min. The minimum amount of radioactivity measurable with the apparatus was considered to be that which gave rise to a deflexion of 50 mm. when the galvanometer was operated at its maximum sensitivity. A $\beta$-ray standard (prepared from uranium oxide) with an activity of 0·01 $\mu$c., produced a deflexion of 250 mm.

*$^{35}S$ standards.* The $^{35}S$ used in the present work was received in the form of pure mustard gas (m.p. 14·2–14·4°). The $^{35}S$ content of 5 mg. of this mustard gas was considered arbitrarily to be 1000 units. The number of atoms of $^{35}S$ in this material was a very small fraction of the total number of S atoms, and as the S content of non-radioactive mustard gas is 20·2%, 1 unit corresponded closely to the $^{35}S/\mu$g. of mustard gas S.

In order to compensate for variations in the sensitivity of the apparatus used for radioactivity measurements, and to avoid making corrections for decay, standard $^{35}S$ samples were measured on each half day that the radioactivity of unknown samples was measured. Each standard consisted

* The use of this apparatus was suggested to us by Dr N. B. Keevil, Department of Physics, University of Toronto, and with his guidance the apparatus was assembled by one of us (M. E.).

of 1 mg. S in the form of benzidine sulphate, and it contained a known number of units of $^{35}S$, i.e. it contained the $^{35}S$ from a known weight of the parent specimen of radioactive mustard gas.

*Relationship between galvanometer deflexion and the $^{35}S$ content of the benzidine sulphate sample.* The ionization chamber apparatus was adjusted so that, as far as possible, a linear relationship existed between the galvanometer deflexions and the activities of the samples. The galvanometer deflexions produced under these conditions by a series of $^{35}S$ standards are shown in Table 1.

Table 1. *Galvanometer deflexions produced by a series of $^{35}S$ standards*

| $^{35}S$ in sample (units) | Galvanometer shunt | Galvanometer deflexion (mm.) |
|---|---|---|
| 0·005 | 1 | 84 |
| 0·010 | 1 | 167 |
| 0·020 | 1 | 330 |
| 0·050 | 0·1 | 72 |
| 0·100 | 0·1 | 141 |
| 0·200 | 0·1 | 296 |
| 0·50 | 0·01 | 91 |
| 1·00 | 0·01 | 190 |
| 2·00 | 0·01 | 433 |

When the data in Table 1 are plotted it is seen that an almost straight-line relationship existed between the amount of $^{35}S$ and the galvanometer deflexions for those measurements in which the 1 or 0·1 shunt was used. There was divergence from such a relationship, however, for the data obtained with the samples which required the use of the 0·01 shunt.

*Self-absorption of β particles by benzidine sulphate samples.* The low energy of the radiation emitted from $^{35}S$ results in absorption of an appreciable fraction of the β particles by the benzidine sulphate. In order to determine the influence of the thickness of the sample on a radioactivity measurement, samples of benzidine sulphate were prepared which contained the same amount of $^{35}S$, but which differed in weight. The experiments were carried out in quadruplicate and all the samples were deposited on Petri dishes 9 cm. in diameter. The results obtained when the radioactivity of these samples was measured are shown in Table 2.

Table 2. *Self-absorption of β particles by benzidine sulphate samples*

| S in sample (mg.) | Benzidine sulphate (mg.) | Average 'thickness' of sample (mg./sq.cm.) | Observed radioactivity* |
|---|---|---|---|
| 1 | 8·8 | 0·14 | 1·00 |
| 2 | 17·6 | 0·28 | 0·95 |
| 3 | 26·4 | 0·41 | 0·91 |
| 4 | 35·2 | 0·55 | 0·86 |
| 5 | 44·0 | 0·69 | 0·81 |

* Expressed as a fraction of the radioactivity of the samples which contained 1 mg. S.

All standard $^{35}S$ samples contained 1 mg. S in the form of benzidine sulphate (8·8 mg.), and in order to take self-absorption effects into consideration, radioactivity measurements were always referred to this amount of S. Since benzidine sulphate samples of unknown radioactivity always contained at least 1 mg. S and were weighed, it was possible by the use of a graph of the data in Table 2 to correct for self-absorption effects in the measurement of $^{35}S$. This correction was usually small, for the S content of the unknown samples rarely exceeded 2 mg.

### *Application of the method*

*Gravimetric determination of sulphur.* The Carius oxidation-benzidine sulphate method was applied to the gravimetric determination of sulphur in a series of organic compounds including mustard gas, its sulphoxide and sulphone, and thiodiglycol. The results obtained in the gravimetric analyses are shown in Table 3.

Table 3. *Gravimetric determination of sulphur in organic compounds by the Carius oxidation-benzidine sulphate method*

| Compound | Wt. of compound analyzed (mg.) | Sulphur | | |
|---|---|---|---|---|
| | | Calc. (%) | Found (%) | Recovery (%) |
| L-Cystine | 6·36 | 26·7 | 27·2 | 101·9 |
| | 5·92 | — | 27·2 | 101·9 |
| Phenylmercapturic acid | 13·57 | 13·4 | 13·7 | 102·2 |
| | 3·19 | — | 13·6 | 101·5 |
| *p*-Fluorophenyl-L-cysteine | 6·90 | 14·9 | 15·2 | 102·0 |
| | 8·64 | — | 15·2 | 102·0 |
| *p*-Fluorophenylmercapturic acid | 8·78 | 12·5 | 12·6 | 100·8 |
| | 8·94 | — | 12·7 | 101·6 |
| Di-2-chloroethyl sulphide | 5·17 | 20·2 | 20·6 | 102·0 |
| | 5·20 | — | 20·5 | 101·6 |
| Di-2-chloroethyl sulphoxide | 7·08 | 18·3 | 19·0 | 103·8 |
| | 7·46 | — | 18·7 | 102·2 |
| Di-2-chloroethyl sulphone | 6·66 | 16·8 | 17·5 | 104·2 |
| | 9·16 | — | 17·2 | 102·3 |
| Di-2-hydroxyethyl sulphide | 10·29 | 26·2 | 26·5 | 101·1 |
| | 12·98 | — | 26·6 | 101·5 |

The results obtained show that in every analysis the weight of the benzidine precipitate was slightly higher than the theoretical amount. This was not due to the presence of sulphur in the reagents for tests showed that, in the quantities used, the reagents did not contain measurable amounts of sulphur. It seems probable that the error was due to occlusion, for McKittrick & Schmidt (1945) have found that this type of error is liable to occur in the gravimetric determination of sulphate as its benzidine salt. Provided there is complete precipitation of the radioactive sulphate when $^{35}S$ is being measured (see below), the occlusion of small amounts of material by the benzidine sulphate is, however, of no significance when no other source of radioactivity is present.

Although the method was not developed for the determination of the total sulphur content of tissues and other biological material, the gravimetric procedure was applied to rat tissues in order to find the amounts of benzidine precipitate which these tissues might be expected to yield in the course of the measurement of their $^{35}S$ content. Four male white rats (body wt. 160–185 g.) were used for the experiments, and the results expressed as mg. of sulphur (mean ± S.E.)/g. of fresh tissue were as follows: liver, 2·55 ± 0·16; kidney, 2·47 ± 0·11; heart, 2·89 ± 0·17; lung, 2·10 ± 0·09; spleen, 2·20 ± 0·18; brain, 1·69 ± 0·09; eye, 2·83 ± 0·12; stomach, 2·12 ± 0·10; small intestine, 2·48 ± 0·09; testis, 1·40 ± 0·05; leg muscle, 2·47 ± 0·09; skin (clipped), 2·25 ± 0·09; carcass (all of the animal that remained after removal of the above tissues), 2·29 ± 0·04.

For accurate results in the determination of sulphate as the benzidine salt, it is necessary to remove phosphate as a preliminary step (Fiske, 1921; Owen, 1936; McKittrick & Schmidt, 1945). The interfering action of phosphate under the conditions used in the present work was shown by experiments in which 1 mg. of sulphur (as $Na_2SO_4$) was precipitated in the presence of 1, 2, 3, 4 or 5 mg. of phosphorus (as $KH_2PO_4$). The weights of the benzidine precipitates corresponded to 1·05, 1·07, 1·11, 1·32 and 1·86 mg. of sulphur, respectively. These results suggest that the gravimetric determination of total sulphur in biological material by this method may give results which are more than 10% in error when the amount of phosphorus present in the material analyzed is three or more times that of the sulphur present. It is noteworthy, however, that the total phosphorus content of a series of rat tissues (liver, kidney, spleen, small intestine, testis and leg muscle) has been found to range from 2·1 to 2·9 mg./g. of fresh tissue (McCarter, unpublished). It appears, therefore, that in the analysis of these tissues, any interference by phosphorus in the determination of total sulphur as benzidine sulphate is likely to be small.

*Measurement of* $^{35}S$. The following experiments were made to gain information about the efficiency of the procedure used for separating radioactive sulphate as its benzidine salt.

Four 5 mg. samples of radioactive mustard gas were oxidized by the Carius method and the sulphate was precipitated as the benzidine salt. In two of these experiments 1 ml. of benzidine reagent was used, and in the other two experiments 2 ml. The precipitates were separated and washed in the usual way and their $^{35}S$ contents were measured. The supernatant liquids obtained after separating and washing the precipitates were collected, however, instead of being discarded. The two supernatant liquids from each precipitate were combined, concentrated and transferred to a Petri dish. The concentrate was then evaporated to dryness and the radioactivity of the residue was measured. Four similar experiments in which rat skin or liver was oxidized together with the mustard gas were also made.

The results (Table 4) show that the fraction of the radioactive sulphate which was not separated in the benzidine precipitate was negligible, whether the volume of benzidine reagent used was 1 or 2 ml. The results also show that the residue from the oxidation of skin or liver did not interfere with the precipitation of the radioactive sulphate.

Table 4. *Completeness of separation of radioactive sulphate as its benzidine salt*

| Material oxidized with 5 mg. radioactive mustard gas | Vol. of benzidine reagent (ml.) | Unseparated $^{35}S$ (% of $^{35}S$ separated) |
|---|---|---|
| — | 1 | <0·3 |
| — | 1 | 0·4 |
| — | 2 | 0·3 |
| — | 2 | 0·5 |
| Skin (0·363 g.) | 1 | <0·3 |
| Liver (0·406 g.) | 1 | 0·3 |
| Skin (0·343 g.) | 2 | <0·3 |
| Liver (0·369 g.) | 2 | <0·3 |

Experiments were conducted in which the recovery of $^{35}S$ was determined when rat tissues (skin, leg muscle, liver, blood) and excreta in amounts similar to those analyzed in tracer studies were oxidized together with weighed amounts of radioactive mustard gas. Four analyses were carried out with each kind of biological material and the results obtained are given in Table 5.

Table 5. *Recovery of* $^{35}S$ *added in the form of mustard gas to rat tissues and excreta*

| Material | Radioactive mustard gas (mg.) | Recovery of $^{35}S$ (%, mean ± S.D.) |
|---|---|---|
| Skin (0·298–0·316 g.) | 5·06–5·26 | 98 ± 1·3 |
| Leg muscle (0·300–0·324 g.) | 5·06–5·16 | 100 ± 2·6 |
| Liver (0·295–0·305 g.) | 5·07–5·15 | 99 ± 2·6 |
| Blood (0·20 ml.) | 4·84–5·12 | 100 ± 2·0 |
| Faeces (0·199–0·297 g.) | 4·99–5·21 | 95 ± 2·7 |
| Urine (0·20 ml.) | 5·07–5·21 | 101 ± 2·7 |

In order to determine the recovery of $^{35}S$ when a carcass was analyzed, two experiments were carried out in each of which a rat weighing 165 g. was killed, 5 mg. of radioactive mustard gas were applied to its skin, and the analysis was completed in the manner described earlier in the paper; the recoveries of $^{35}S$ were 98 and 96 %.

## DISCUSSION

The Carius oxidation-benzidine sulphate method is well suited to the routine measurement of $^{35}S$ in biological material, for the operations involved are simple, require little time, and are such that they can be conducted simultaneously with groups of analyses. A possible objection to the Carius procedure, namely that loss of material is liable to occur as a result of explosion during the oxidation process, is of no significance, for under the experimental conditions described herein, the explosion of a bomb is a very rare occurrence. The benzidine sulphate precipitate can readily be collected in a uniform manner, and although the procedure employed in the present work for preparing the precipitate for radioactivity measurement was designed for use with an ionization chamber apparatus, it can easily be adapted to meet the requirements of other forms of apparatus suitable for the measurement of the radiation from $^{35}S$.

The method is applicable to the gravimetric determination of sulphur in a variety of organic compounds (see Table 3). This, in itself, is insufficient, however, to justify the conclusion that the method permits the quantitative recovery of $^{35}S$ present in such compounds. The weight of the final precipitate in a gravimetric analysis may correspond to the calculated weight as a result of a cancellation of errors, e.g. incomplete precipitation may be offset by occlusion. This possibility can be excluded, however, since it has been found that no significant amount of $^{35}S$ remains behind after separation of the benzidine sulphate (Table 4). This finding, taken in conjunction with the results of the gravimetric analysis of mustard gas, shows that with the present method the separation of the $^{35}S$ of radioactive mustard gas as benzidine sulphate is essentially quantitative. Under these conditions radioactive mustard gas can be used as a source of $^{35}S$ in studies of the accuracy of the method as a means of measuring $^{35}S$ in biological material. This was done by comparing the amounts of $^{35}S$ found when samples of a given specimen of radioactive mustard gas were analyzed alone, and when they were analyzed in the presence of biological material. The results reported in Table 5 show that in a total of twenty-four analyses in which such experiments were carried out with six different kinds of biological material, the mean recovery of $^{35}S$ was 99 %, and the standard deviation of a single measurement from the mean was 2·8 %. The destructive oxidation of mustard gas does not occur readily, and this fact, when considered together with the findings described above, suggests that the present method is suitable for the measurement of $^{35}S$ in a variety of biological materials.

## SUMMARY

1. A method for the measurement of radioactive sulphur ($^{35}S$) in biological material is described. A sample of the material is oxidized by the Carius method and the sulphate is precipitated as benzidine sulphate. The radioactivity of the benzidine sulphate is measured in an ionization chamber operated in conjunction with an FP-54 valve amplifying system and a galvanometer, and is compared with the activities of samples of benzidine sulphate prepared from the parent radioactive specimen.

2. When mustard gas prepared from radioactive sulphur was analyzed in the presence of biological material (skin, leg muscle, liver, blood, faeces, or urine) by this method, the mean recovery of $^{35}S$ was 99 %, and the standard deviation of a single measurement from the mean was 2·8 %.

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# A Method for the Colorimetric Micro-estimation of Thymine

By D. L. WOODHOUSE, *Cancer Research Laboratory, Medical School, University of Birmingham*

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Thymine is the distinctive pyrimidine of the nuclear deoxyribosenucleic acid. A sensitive method for its estimation would be of service in the analysis of nucleic acid, in conjunction with other analytical techniques. Thymine can be distinguished from uracil or cytosine by the stable red colour which is produced when it is coupled with the diazo reagent of Koessler & Hanke (1919) in a solution made alkaline with sodium carbonate and afterwards reduced with hydroxylamine in presence of sodium hydroxide (Hunter, 1936). This reaction has been examined as regards sensitivity, specificity and suitability for quantitative work.

## EXPERIMENTAL

### *Micro-estimation of pure thymine*

*Reagents and procedure.* Slight modifications were made of Hunter's (1936) procedure. The *diazo reagent* used was that of Koessler & Hanke, viz. (i) 1·5 ml. sulphanilic acid solution (4·5 g. in 45 ml. conc. HCl, made up to 500 ml. with water), and (ii) 1·5 ml. $NaNO_2$ solution (25 g. in 500 ml. water). Solutions (i) and (ii) are quickly mixed and cooled; after 5 min., 6 ml. $NaNO_2$ solution are added and the solution made up to 50 ml. It is kept cold in ice water, and the reagent used not less than 20 min. after making the final dilution. If kept cold it is stable for 24 hr. and can conveniently be prepared daily from the stock solutions.

*Coupling* was performed at 20° and allowed to proceed for exactly 20 min., after which the NaOH was added, followed by hydroxylamine. During the coupling, the tubes should be immersed in a water bath at the prescribed temperature. The extension of time as compared with Hunter's qualitative test (5 min.) gives a more intense colour, but no extra tint in similarly conducted blank tests in which distilled water replaces the thymine solution. It is advisable to use stoppered vessels to exclude $NH_3$ or acid fumes, and to mix the solutions quickly and thoroughly at all stages.

Intensity and stability of the colour was measured by means of a Spekker absorptiometer, using the following amounts of reactants, mixed in the order given: $Na_2CO_3$ (2·0 ml.; 2·4 (w/v)% solution of anhydrous salt); distilled water (2·0 ml.); diazo reagent (2·0 ml.); thymine solution (2·0 ml. containing 0·0125–0·100 mg./ml.); NaOH (2·0 ml. of 3N); hydroxylamine hydrochloride (0·2 ml. of 20% w/v).

Colour values were read using a blue filter (Chance OB2). Beer's law was obeyed up to thymine concentrations of 0·1 mg./ml. Curves have been made with specimens of thymine kindly given by Genatosan Ltd. (Loughborough) and Schwarz Laboratories Inc. (New York). The samples gave identical readings.

The time of coupling must be measured exactly and the temperature should not vary by more than ±0·5°. If the reaction is carried out at a higher temperature, e.g. 45°, more intense colours are produced even with very dilute solutions; 10 $\mu$g. in a final volume of 10 ml. can be detected with ease, but these conditions are not recommended for quantitative estimations.

*Substances reacting under similar conditions.* (i) When tested by the above technique none of the purines or pyrimidines known to occur in nucleic acids gives the pink colour except thymine. The other bases yield yellow tints so that if relatively large amounts of these substances are present together with thymine, they affect the colour produced. The presence of uracil in the proportion of 0·1 mg. to 0·1 mg. thymine occasions little error, however, when the prescribed conditions are observed.

(ii) The reaction yields yellow colours of various intensities with amino-acids or proteins at concentrations of 0·2 mg./ml. With cystine a tint very similar to the thymine colour is produced.

(iii) Certain aldehydes, including furfural, and decomposition products produced by mild hydrolysis of nucleic acids, produce pink tints so that precautions must be taken to exclude such substances when estimating thymine. In these circumstances it was not found possible to apply the method directly to hydrolysates of nucleic acid.

*Comparison with the method of Harkins & Johnson* (1929). These writers described a test for thymine in which the

acetol obtained by refluxing brominated thymine with $Ba(OH)_2$ is distilled and combined with a reagent produced by heating *o*-nitrobenzaldehyde with $FeSO_4$ in presence of concentrated $NH_3$ followed by steam distillation. After acidifying the mixture with HCl and then making alkaline with $NaHCO_3$, a blue fluorescence results which is more intense when viewed in the mercury arc light.

I have confirmed the statement that, by this technique, 1 mg. thymine can be detected if the volume of the completed test is reduced to 1 ml., but experiments showed that the Hunter (1936) test was much more delicate. Also the 'diazo coupling' method requires only simple apparatus and is much more convenient for routine analyses.

### *Application of the method to nucleic acid*

#### (*a*) *Technique*

The Hunter (1936) reaction cannot be applied directly to a solution of hydrolyzed nucleic acid, since certain reaction products, particularly from ribosenucleic acid, also produce red-coloured compounds. Efforts to eliminate these, or to extract the thymine quantitatively with various solvents, were not successful. It was, however, found possible to apply the reaction after separating the pyrimidine as a silver compound. The method was based partly on the technique of Levene (1922) for separation of purines, and partly on that of Barnes & Schoenheimer (1943) for the isolation of thymine from thymus nucleic acid. Levene showed that, when HCl gas is passed freely for some time into a suspension of nucleic acid in methanol, heat is liberated, the nucleic acid dissolves and the purines are precipitated. After standing in the cold overnight, they can be removed by centrifugation. Barnes & Schoenheimer evaporated such a supernatant from the purine precipitate to a small volume, hydrolyzed the products by 20% HCl in a sealed tube, and isolated some crystalline thymine.

In the present work the hydrolysate from 50 mg. of deoxyribosenucleic acid was evaporated to dryness under reduced pressure on the water bath and free HCl removed as thoroughly as possible. The residue was dissolved in 3 ml. of 25% (v/v) $H_2SO_4$, 1·5 ml. transferred to a combustion tube (5–6 in. long) and heated in a furnace at 175° for 2·5 hr. Sulphates and phosphates were removed by adding hot, saturated $Ba(OH)_2$ to pH 9 and centrifuging. The precipitate also carried down all coloured carbonized materials. The supernatant liquid was concentrated to about 10 ml., acidified with $HNO_3$ to pH 6 and 1 ml. of 5% (w/v) $AgNO_3$ added—shown to be a slight excess by testing externally with $Ba(OH)_2$ solution. The silver-purine compounds, together with any remaining AgCl, were removed on the centrifuge after standing in the cold. The supernatant was then made alkaline to pH 9 with cold $Ba(OH)_2$, added drop by drop. A flocculent precipitate resulted, consisting of pyrimidine-silver salts together with some silver hydroxide, which, on standing, gave a brownish appearance to the precipitate. After chilling overnight, the mixture was centrifuged, the precipitate washed with a few drops of distilled water, heated to 80° with 5 ml. of N-HCl to decompose the Ag compounds, and, after cooling, the insoluble AgCl was removed by centrifuging. The solution containing the thymine was made up to 10 ml., solid $Na_2CO_3$ added until effervescence ceased, the volume adjusted to 25 ml., and 2 ml. portions were submitted to the modified Hunter test.

#### (*b*) *Thymine in commercial nucleic acids*

(i) *Thymus nucleic acid.* Samples of 20 and 50 mg. of the material were subjected to the procedure outlined above, and the results compared with the P content as determined by Holman's (1943) method. The latter gave a figure of 7·3% P as compared with the theoretical of 9·2% for the tetrasodium salt of deoxyribosenucleic acid, indicating 80% purity.

Colorimetric estimation of thymine showed a content of 0·128 mg. in 20 mg. of nucleic acid corresponding to 70% of theory, or 90% of the expected amount based on the P content.

(ii) *Yeast nucleic acid.* Repeated analyses of such material demonstrated that only a trace of thymine was present, the final colour being tinged only very slightly pink. The negative diphenylamine reaction (Dische, 1930) obtained with the sample supported the assumption that the deoxyribosenucleic acid content was very small.

#### (*c*) *Thymine in nucleic acid samples from fresh calf thymus*

These were prepared by a modification of Hammarsten's (1924) method. In order to remove the cytoplasmic constituents the minced tissue was extracted three times with physiological saline which removes a high proportion of the cytoplasmic nucleoprotein without affecting the nuclear nucleoprotein (Mirsky & Pollister, 1946). The isolation of the nucleic acid was then completed as described by Hammarsten (1924). This method, which yields fibrous nucleic acid, was used since it does not require a high-speed centrifuge such as must be employed to clarify the highly viscous solutions of nucleoprotein obtained in Mirsky's method of extraction with M-NaCl solution.

The P content of the product was 7·2%, which agreed closely with the value of 7·09% obtained by Hammarsten (1924), but the colour obtained in the quantitative diphenylamine reaction (Dische, 1930; Siebert, 1940) was definitely greater than that given by commercial thymus nucleic acid. When solutions of the same P content were compared, a ratio of 1·13 : 1·00 was found. The thymine content was 7·05%. These figures suggest that the material was probably free from ribosenucleic acid but still retained some impurity. In order to remove this a solution in 2N-NaOH was warmed on a water bath for 1 hr. at 70° and rendered faintly acid with acetic acid. The precipitate was dissolved in 0·1N-NaOH, reprecipitated from ethanol, washed several times with ethanol-ether and dried in a desiccator to constant weight.

Portions on analysis gave the following values: (i) P content 8·8% (cf. theoretical 9·18% and for commercial specimen 7·57%); (ii) Dische diphenylamine reaction colour relative to an equivalent solution of commercial nucleic acid was 1·25 : 1·00; (iii) N content 15·2% (theory 15·6%); (iv) thymine content 9·0% (theory 9·3%); (v) as a further check on the deoxyribose content compared with the commercial specimen, the cysteine reaction (Stumpf, 1947) was carried out, yielding a relative colour value of 1·20 : 1·00.

The material thus appears to have been 96% deoxyribosenucleic acid, and the method for thymine estimation, which has been described, is satisfactory for applying to nucleic acid in quantities as small as 20 mg.

Further work is being done to compare the thymine content of deoxyribosenucleic acid isolated from animal tumours with that derived from normal tissues.

SUMMARY

1. A colorimetric method is described for the quantitative estimation of thymine which is suitable for quantities between 50 and 200 $\mu$g. in 2 ml. of solution.

2. The application of this method to the estimation of thymine in 50 mg. samples of deoxyribosenucleic acid is reported.

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# Anaerobic Leakage of Potassium from Brain

By K. C. DIXON, *Department of Pathology, University of Cambridge*

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Interruption of blood supply to tissues and organs is one of the main causes of organic disease. Characteristic histological changes follow such vascular occlusion, and it seems likely that these have their origin in chemical and metabolic disturbances produced by deprivation of oxygen and food substances from the tissue cells. The present communication describes a study of some of the chemical events taking place when the supply of oxygen and glucose to the cells of the brain is interrupted. These two deficiencies are probably the immediate determinants in the dislocation of cerebral function following vascular accidents, and thus may be regarded as the primary factors in ischaemic damage.

The intracellular concentration of potassium is many times greater than that outside the cells. Danowski (1941) showed that this intracellular potassium diffuses out of red blood cells when the blood sugar is used up, or when glycolysis is inhibited by fluoride. Harris (1941) reported similar results. Dickens & Greville (1933) found that brief anaerobic incubation of slices of cerebral cortex, without substrate, causes inhibition of glycolysis, and Macfarlane & Weil-Malherbe (1941) ascertained that pyrophosphate is destroyed at the same time. Colfer & Essex (1947) observed a loss of potassium from brain during convulsions. Potassium ions are known to cause intense aerobic stimulation and anaerobic inhibition of the glycolytic metabolism of cerebral cortex (Ashford & Dixon, 1935; Dickens & Greville, 1935). For these reasons the present work was designed to find out if leakage of potassium also occurs from brain when deprived of oxygen and glucose. In this tissue a rise in the external concentration of potassium might produce profound metabolic changes in neighbouring areas outside the initial region of ischaemia as well as affecting radically the internal milieu of the cells primarily affected.

## METHODS

Slices of rabbit cerebral cortex were suspended in bicarbonate-Ringer solution without glucose made up according to Dickens & Greville (1935) and previously saturated with $N_2$ containing 5% $CO_2$. After washing in this solution, the slices were transferred to Warburg manometer cups containing 1·5 ml. of fluid. In general, half the slices were incubated in bicarbonate-Ringer without substrate, and the remaining slices in the same volume of the same salt solution containing in addition 0·2% glucose. In some experiments half the slices were incubated in this glucose-Ringer, and the remainder in the same glucose-Ringer containing 0·05% NaF. In a few experiments three samples of the slices were used, one without substrate, one with glucose and one with glucose and fluoride. The manometers were filled at room temperature with $N_2$ containing 5% $CO_2$ the gas being passed through for 3–3·5 min.

*Potassium* estimations were made as follows. Practically the whole of the 1·5 ml. of fluid remaining in the manometer cups after incubation at 38° was pipetted away from the slices into centrifuge tubes using Pasteur pipettes. This fluid was then centrifuged at high speed for 15 min. and 1 ml. of the clear supernatant fluid was pipetted off into Pyrex crucibles and evaporated to dryness in an oven at 100–120°. Conc. $HNO_3$ (0·5 ml.) was then added to each, and again the crucibles were heated to dryness. The crucibles were then incinerated for 3 hr. in an electric muffle furnace at 400–450°, to destroy ammonium salts.

The colourless residues were extracted twice with 0·5 ml. portions of 0·1 N-HCl and twice with 0·5 ml. portions of distilled water. During extraction the crucibles were briefly heated, each time on a hot plate, till steaming commenced. The four washings from each crucible were transferred, using Pasteur pipettes, to numbered graduated centrifuge tubes and each made up to 2 ml. (a little fluid is lost by evaporation during the heating). Sodium cobaltinitrite solution (1 ml.; see King, 1946) was added to each tube, and after standing for 45 min. the precipitated potassium cobaltinitrite was separated by centrifugation. After draining and washing twice with 70% ethanol, the cobalt in the precipitate was estimated by the method of Jacobs & Hoffman (1931) using the colour reaction with choline chloride and $Na_4Fe(CN)_6$. The volume at the final stage was made up to 9 ml., and the colours were compared with similarly treated standard K and standard Co solutions in an Evans photoelectric colorimeter, as designed by King (1946), using an Ilford tricolor red filter as suggested by this author. A sample of the Ringer solution used in the experiment was included in each batch of estimations as a check on recovery following incineration. The recovery of K from this bicarbonate saline was always at least 90 and often 100% (see Table 1). Equivalent amounts of KCl alone cannot be estimated in this way as recovery is extremely poor, presumably owing to unshielded K salts attacking the Pyrex crucibles above 400°. In the ashed bicarbonate-Ringer, on the other hand, only a minute fraction of the contained K salts probably comes into sufficiently intimate contact with the Pyrex to cause appreciable loss. It will be evident from the figures given that the recovery of K contained in the incinerated bicarbonate saline is practically complete using the method described above.

*Anaerobic glycolysis* ($Q_L^{N_2}$) was measured manometrically. Readings were taken every 10 min. and the values of $Q_L^{N_2}$ were calculated over the first 0·5 hr.

Considerable uniformity in the values of anaerobic glycolysis was shown with different specimens of rabbit-brain cortex. Furthermore, glycolysis was fairly constant throughout incubation, only falling by 14% (average of 17 experiments) over 40 min. This constancy contrasts with observations of previous workers using rat brain. For the sake of brevity protocols have had to be omitted.

## RESULTS

Table 1 summarizes the results of estimations of potassium in Ringer solution before and after incubation with brain slices for 1 hr. The mean dry weights of tissue used are also shown. Considerable increase in the potassium concentration of the fluid surrounding the tissue was observed, when the medium was devoid of glucose, or contained fluoride as well as glucose. With glucose alone, on the other hand, this increase was eliminated and was generally replaced by slight diminution in the potassium concentration.

A clearer picture of these results is given in Table 2 by evaluating the amounts of potassium liberated or absorbed per unit dry weight of tissue. Mean values for anaerobic glycolysis are also shown. The amount of potassium liberated or absorbed is expressed in $\mu$l., on the basis of 1 $\mu$equiv. K = 11·2 $\mu$l. The number of $\mu$l. of potassium liberated or absorbed/mg. dry wt. of tissue/hr. gives values of convenient order. A positive value signifies liberation of potassium and a negative value a corresponding absorption. Owing to stringency of space individual values have had to be omitted. In every case except one the results obtained refer to parallel experiments under at least two of the conditions studied with the tissue of each of the rabbits used.

From Table 2 we see that, in the absence of glucose, potassium was in every case liberated into the fluid surrounding the brain tissue, at an average rate of 2·2 $\mu$l./mg./hr. The leakage of potassium was suppressed by glucose and was usually replaced by a small net absorption of potassium (0·4 $\mu$l./mg./hr.). In the presence of glucose the anaerobic glycolysis was high ($Q_L^{N_2} = 28 \pm 3$), but in the absence of glucose very small ($Q_L^{N_2} = 2 \pm 1$). It appears that when glycolysis can take place the amount of

Table 1. *Potassium leakage from brain tissue*

(Showing K concentration in Ringer before and after anaerobic incubation for 1 hr. at 38° with slices of rabbit-brain cortex. The slices were suspended in 1·5 ml. of fluid. Observations on the tissues of 22 rabbits are included.)

| | | Initial Ringer solution | Initial Ringer after incineration | Ringer after incubation with brain slices in the presence of: Glucose 0·2% | No substrate | Glucose 0·2%, NaF 0·05% |
|---|---|---|---|---|---|---|
| K concentration m.equiv./l. | Mean | 2·45 | 2·37 | 2·08 | 3·86 | 3·62 |
| | Range | 2·3–2·6 | 2·1–2·6 | 1·7–2·6 | 3·1–4·9 | 3·1–3·9 |
| | S.D. | 0·11 | 0·14 | 0·26 | 0·48 | 0·23 |
| | S.E. of mean | 0·03 | 0·03 | 0·06 | 0·12 | 0·07 |
| Dry weight of tissue used (mg.) | Mean | — | — | 13·48 | 11·43 | 11·22 |
| | Range | — | — | 8·1–20·2 | 7·7–17·3 | 8·1–16·3 |
| | S.D. | — | — | 3·51 | 2·99 | 3·15 |
| No. of determinations on initial solutions | — | 13 | 16 | — | — | — |
| No. of rabbits from which tissue was used | — | — | — | 21 | 16 | 10 |

Table 2. *Potassium leakage from brain in terms of dry weight*

(K liberation into the fluid bathing the brain slices during incubation for 1 hr. at 38° is expressed as μl. of K/mg. dry wt. of tissue. Positive values indicate liberation of K, negative values indicate absorption. 1 μequiv. of K = 11·2 μl. The anaerobic glycolysis is also shown. The figures are from the same series of experiments on the tissues of 22 rabbits referred to in Table 1.)

| | μl. K/mg. dry wt./hr. | | | $Q_L^{N_2}$ | | |
|---|---|---|---|---|---|---|
| | Glucose 0·2% | No substrate | Glucose 0·2%, NaF 0·05% | Glucose 0·2% | No substrate | Glucose 0·2%, NaF 0·05% |
| Mean | −0·41 | +2·24 | +1·89 | 27·7 | 1·6 | 1·9 |
| Range | −1·0 to +0·4 | +1·7 to +3·2 | +1·2 to +2·5 | 23–32 | 0–4 | 1–3 |
| S.D. | 0·36 | 0·38 | 0·42 | 2·6 | 1·0 | 0·9 |
| S.E. of mean | 0·08 | 0·10 | 0·13 | 0·6 | 0·3 | 0·3 |
| No. of rabbits | 21 | 16 | 10 | 22 | 15 | 10 |

potassium leaving the cells was more than compensated by increased entry, so that there was usually no net loss of potassium from the cells into the surrounding fluid, and often there was an uptake from the fluid by the cells. In the absence of glycolyzable substrate, however, there was always a highly significant loss of potassium from the cells into the surrounding fluid.

Table 2 also shows that glycolysis was practically eliminated by fluoride ($Q_L^{N_2} = 2 \pm 1$). Danowski (1941) found that the addition of fluoride produced leakage of potassium from human red cells even when blood sugar was present. The results of Table 2 amply confirm this for rabbit brain. When glycolysis was suppressed by fluoride, potassium leaked out into the surrounding fluid just as in the absence of glucose at the rate of 1·89 μl./mg. dry wt./hr. In each parallel experiment using glucose alone this leakage was prevented and was often replaced by the actual removal of potassium from the fluid. The suppression of potassium leakage was not due to the mere static presence of glucose, but to its dynamic utilization in supplying energy to the glycolyzing cell, since when glycolysis was inhibited leakage again became evident.

In the absence of glycolyzable substrate appreciable potassium leakage occurred even in half an hour. This is apparent from Table 3 which records observations with brain tissue of two rabbits incubated for only half an hour; in these two experiments about 1·0 μl. K/mg. dry weight was liberated in half an hour. Approximately twice this amount (average 2·2 μl.) leaked out in 1 hr. (Table 2). In Table 3 the effect of glycolyzable substrate in curbing leakage and promoting uptake is also evident.

Table 3. *Potassium accumulation/mg. dry weight in fluid incubated with brain slices for* 0·5 *hr.*

| Rabbit no. | μl. K liberated/mg. dry wt./0·5 hr. | | |
|---|---|---|---|
| | Glucose | No substrate | Glucose-fluoride |
| 10 | −1·0 | +1·0 | +1·1 |
| 11 | −1·0 | +1·0 | — |

Some potassium leakage may even occur while setting up the experiments at room temperature. Thus in three cases Ringer solution without substrate or with glucose-fluoride was treated with brain slices, and equilibrated with nitrogen/carbon dioxide under the same conditions as in the actual experiments up to the point of the start of incubation. Then, instead of being placed in the bath at 38°, the fluid was removed from the tissue slices for analysis without incubation. In two cases without substrate the potassium concentration of the fluid had risen so that 0·9 and 0·6 μl. of potassium were liberated per mg. dry weight. In the third case with glucose-fluoride, however, no increase was detected. After incubation for 1 hr., tissue from these three rabbits liberated 2·0–2·2 μl. of K.

When both glucose and oxygen were supplied to the cells (just as with glucose alone) no net leakage of potassium occurred, and the uptake of potassium from the fluid by the tissue is apparently even more definite than with glucose alone. Thus with tissue from two of the rabbits parallel experiments were set up with glucose and oxygen/carbon dioxide as well as the anaerobic experiments recorded in Table 2. In one case with glucose alone the rate of disappearance of potassium was −0·6 μl./mg. dry wt./hr., but with glucose and oxygen this was −1·0 μl./mg. dry wt./hr., while in the absence of both glucose and oxygen, there was a net accumulation of potassium at the rate of +1·7 μl./mg. dry wt./hr. Similarly, in the other case, with glucose alone, the disappearance of potassium was −0·6 μl./mg. dry wt./hr., but with glucose and oxygen this was −1·2 μl./mg. dry wt./hr., whereas the anaerobic accumulation with glucose and fluoride was +2·1 μl./mg. dry wt./hr. It thus appears that glucose and oxygen checked the leakage and promoted uptake of potassium even more effectively than glucose alone.

How far potassium leakage is accompanied by tissue disintegration is uncertain. Lutwak-Mann (1947) found tissue disintegration of gastric mucosa to be promoted by anaerobic substrate deprivation. In the present experiments with brain also more

debris was usually formed in the absence of substrate than when glucose was present. In one experiment an exceptionally large amount of debris was formed without substrate; this was much more marked than with glucose and even more marked than with glucose-fluoride. However, the leakage of potassium was no greater without substrate than with glucose-fluoride. This indicates that potassium leakage is probably not mainly occasioned by mere tissue disintegration, but the two phenomena may well progress together. In the later experiments of this series the spun debris was included in the dry weight determinations.

## DISCUSSION

We may conclude from the results recorded in Tables 1–3 that when both the supply of glucose and oxygen to the brain cells is cut off there is considerable leakage of potassium into the extracellular fluid. This loss of potassium may be one of the basic factors responsible for ischaemic injury to cells, as potassium is doubtless an essential intracellular constituent on whose presence in high concentration many enzymic processes may depend. Furthermore, in the case of brain, it is possible that the loss of potassium from damaged cells may have other consequences since in brain potassium ions have such remarkable effects on metabolism. The irritation and paralysis associated with vascular accidents may thus depend in part on these metabolic effects produced by potassium ions which have emerged from the primary focus of ischaemic damage out into adjacent regions, as well as on the escape of essential intracellular constituents (such as potassium) out of the initially injured tissue.

## SUMMARY

1. During anaerobic metabolism in the presence of glucose there was no increase in the concentration of potassium in the fluid bathing slices of cerebral cortex. In some cases there was a decrease.

2. Under the same conditions, but in the absence of glucose, there was always a significant rise in the potassium concentration of the fluid surrounding the brain slices.

3. The action of glucose in preventing this loss of potassium is dynamic: it depends on the active utilization of glucose by the glycolyzing cell. Where glycolysis was inhibited by fluoride potassium leaked into the environmental fluid just as in the absence of glucose. Brain thus resembles red blood cells for which Danowski (1941) has described a similar effect.

4. The significance of these findings on the nature of ischaemic damage is briefly considered.

I am very grateful to Mr N. T. J. Bailey, Statistician to the Department of Medicine, Cambridge, for checking the figures in Tables 1 and 2, and for useful advice.

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# The Activation of Phosphoglucomutase by Metal Ions

By L. H. STICKLAND
*Department of Experimental Pathology and Cancer Research, University of Leeds*

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The existence of glucose-1-phosphate and its conversion to glucose-6-phosphate in muscle were first described by Cori & Cori (1936). Kendal & Stickland (1938) claimed that $Mg^{++}$ and fructofuranose-1:6-diphosphate were both essential for this conversion, but Cori, Colowick & Cori (1938) stated that 'the contention of Kendal & Stickland that hexosediphosphate acts as a coenzyme and is essential for the reaction is not substantiated by our findings'. They found, on the other hand, that $Mn^{++}$ and $Co^{++}$ are more efficient activators than $Mg^{++}$, and that the enzyme (named by them phosphoglucomutase) shows a large residual activity in the absence of any added metal. This has led to the view, expressed typically by Sumner & Somers (1947), that 'phosphoglucomutase requires no coenzyme'.

The present paper deals with some of the effects of metallic ions on the activity of phosphoglucomutase. In a later publication the author hopes to

describe the role of fructofuranose-1:6-diphosphate (*HDP*), and to reconcile the conflicting statements of Cori *et al.* (1938) and Kendal & Stickland (1938). For the present it is enough to reassert that *HDP* is essential for maximal activity of phosphoglucomutase, as will be seen later in Table 1.

## METHODS

*Phosphoglucomutase.* In most of the experiments the enzyme was partially purified by the method of Colowick & Sutherland (1942), but similar results can be obtained equally well in a simple dialyzed muscle extract.

*Glucose-1-phosphate.* This was prepared by a method substantially the same as that of Sumner & Somers (1943), and was used as the K salt.

*Salts of metals.* The salts used were A.R., where this quality was available (e.g. potash alum, chrome alum and lead acetate); in the other cases ordinary commercial samples were used without special purification.

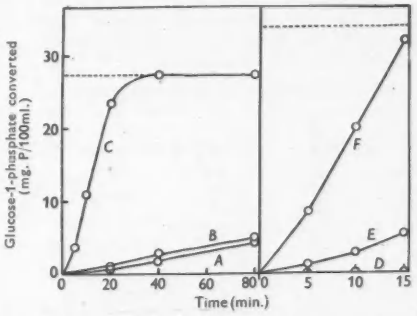


Fig. 1. The course of the conversion of glucose-1-phosphate to glucose-6-phosphate. Curve *A*, $Al^{+++}+HDP$; curve *B*, $Mg^{++}+HDP$; curve *C*, $Al^{+++}+Mg^{++}+HDP$; curve *D*, $Mg^{++}+HDP$; curve *E*, $Mn^{++}+HDP$ or $Cr^{+++}+Mn^{++}+HDP$; curve *F*, $Cr^{+++}+Mg^{++}+HDP$. The dotted lines indicate the amount of conversion at equilibrium. The concentration of *HDP* was 0·0003 M, and that of the metals was optimal (see text).

*Measurement of the course of reaction.* The reaction was followed by determining the residual glucose-1-phosphate by 5 min. hydrolysis in N-HCl at 100°. The reaction mixtures contained the substrate, etc., at the following final concentrations, unless otherwise stated: glucose-1-phosphate, 0·01 M; Na phosphate buffer, pH 7·5, 0·005 M (approx.); *HDP*, 0·0003 M. The various metals and their concentration will be given in the text and tables. For the sake of uniformity, concentrations are expressed in terms of the 'molarity' of cation (e.g. $Cr^{+++}$) and not of the actual salt used (e.g. $Cr_2(SO_4)_3$). The reaction mixtures were made up in lots of 1·0 or 2·0 ml. in 6 × 0·5 in. test tubes, and were placed in the water bath at 38° for some 5 min. before the addition of the enzyme solution (0·1 or 0·2 ml. at a predetermined dilution). The reaction was stopped, usually after 15 min., by the addition of 3 vol. of 4% trichloroacetic acid, and the inorganic $PO_4$ determined in samples of the filtrate, before and after 5 min. acid hydrolysis at 100° (Fiske & Subbarow, 1925). When the time course of the reaction was to be followed, larger volumes of mixture were made, and 1·5 or 2·0 ml. samples withdrawn from each into trichloroacetic acid at zero time and after the required intervals.

The use of a single point to measure relative reaction velocities was justified by the observation that the reaction is almost linear until the equilibrium point is nearly reached. A slight lag in the first quarter of the reaction was sometimes noticed (Fig. 1). To obtain the greatest possible accuracy, the concentration of the enzyme was always adjusted so that, in the fastest reaction of any series, some 75% of the conversion had taken place in the time allowed (15 min.). Finally, the most important points were checked by following the course of the reaction to the equilibrium point.

## RESULTS

The basic observation is that maximal activation of phosphoglucomutase is achieved only with a combination of three components, viz. two metals and *HDP* (Table 1). Of these three components, the present work is concerned only with the two metals, and, in all the experiments that follow, it may be assumed that *HDP* is present at a concentration of 0·0003 M unless the contrary is stated. A wide range of metals has been tested for their power to take the place of either $Mg^{++}$ or $Al^{+++}$ in the system shown in Table 1, and these will be dealt with in turn.

Table 1. *The indispensability of three components for full phosphoglucomutase activity*

(Reaction mixture as described in text. Concentrations of additions: $Mg^{++}$, 0·003 M; $Al^{+++}$, 0·0005 M; *HDP*, 0·0003 M.)

| Additions | Glucose-1-phosphate converted in 15 min. (mg. P/100 ml.) Exp. 1 | Exp. 2 | Exp. 3 |
|---|---|---|---|
| $Mg^{++}+HDP$ | 1·1 | 0·0 | 0·0 |
| $Al^{+++}+HDP$ | 3·1 | 1·3 | 0·0 |
| $Mg^{++}+Al^{+++}$ | 1·6 | 1·7 | 0·7 |
| $Mg^{++}+Al^{+++}+HDP$ | 27·7 | 22·6 | 11·0 |

*Magnesium.* The indispensability of $Mg^{++}$ was demonstrated by the use of a test system containing substrate, enzyme, $Al^{+++}$ and *HDP*. The addition of other metals to this system showed that only $Mg^{++}$ led to the appearance of any considerable activity (Table 2). $Cr^{+++}$ is not included in this table, as it is a special case which will be considered later. The concentrations used, except in the case of $Mg^{++}$, were 0·002 and 0·0002 M, and it is most unlikely that any activity should exist which would not be detected at one or other of these concentrations. $Mg^{++}$ was tested at 0·004 M, the concentration shown to be optimal for glycolysis by Lohmann (1931); Cori *et al.* (1938) observed an ill-defined optimum for phosphoglucomutase at *c.* 0·005–0·01 M.

Of special interest are the negative results with $Mn^{++}$ and $Co^{++}$; these will call for further comment later.

Table 2. *The irreplaceability of* $Mg^{++}$

(Reaction mixture and conditions of experiment as described in the text. Concentrations: $Al^{+++}$ (0·005 M) and *HDP* (0·0003 M) present throughout; $Mg^{++}$, 0·003 M, other metals, 0·002 M. The other metals were also tested at 0·0002 M, with similar negative results. Other metals tested, at 0·002 and 0·0002 M, with completely negative results, were $Be^{++}$, $Ca^{++}$, $Zn^{++}$, $Pb^{++}$, $Hg^{++}$, $UO_2^{++}$, $La^{+++}$, $Bi^{+++}$, $Zr^{++++}$, $Ce^{+++}$.)

| Addition | Glucose-1-phosphate converted in 15 min. (mg. P/100 ml.) 1st series | 2nd series | 3rd series |
|---|---|---|---|
| None | 2·7 | 0·1 | 0·2 |
| $Mg^{++}$ | 26·4 | 21·7 | 12·3 |
| $Fe^{+++}$ | 3·3 | — | — |
| $Mn^{++}$ | 3·2 | — | — |
| $Sr^{++}$ | — | 2·3 | — |
| $Ba^{++}$ | — | 1·7 | — |
| $Cu^{++}$ | — | 2·0 | — |
| $VO^{++}$ | — | — | 1·1 |
| $Ni^{++}$ | — | — | 2·5 |
| $Co^{++}$ | — | — | 1·0 |
| $Ti^{++++}$ | — | — | 1·1 |

The relationship between concentration of $Mg^{++}$ and degree of activity is shown in Fig. 2. The optimum, 0·003 M, agreed closely with that found

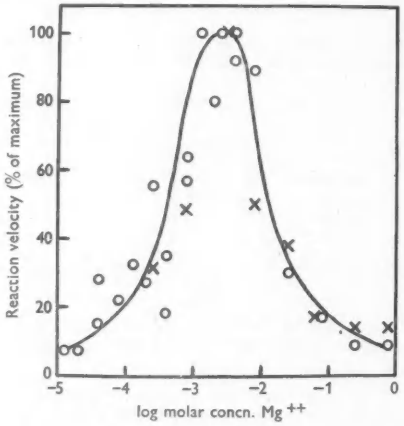


Fig. 2. The relationship between concentration of $Mg^{++}$ and activity of phosphoglucomutase. *HDP* (0·0003 M) present throughout. Circles: in the presence of $Al^{+++}$ (0·0005 M); crosses: in the presence of $Cr^{+++}$ (0·00002 M).

by Lohmann (1931) for the whole glycolytic process, but not so closely with that found by Cori *et al.* (1938) for partially activated phosphoglucomutase.

It was the same whether the second metal were $Al^{+++}$ or $Cr^{+++}$; the optimum for $Mg^{++}$ has not been specially determined for any other metals, and $Mg^{++}$ has been used at 0·003 M in all other experiments.

*The second metal.* In a test system containing substrate, enzyme, $Mg^{++}$ and *HDP*, the inclusion of $Al^{+++}$ led to the appearance of a high enzymic activity (Table 1), and this power was shared by a number of other metals, notably $Cr^{+++}$, $Pb^{++}$, $Fe^{+++}$, $UO_2^{++}$, $Ce^{+++}$, $Ti^{++++}$, $Be^{++}$, etc. (Table 3).

Table 3. *Metals which can supplement* $Mg^{++}$

(Reaction mixture and conditions of experiment as usual. Concentrations: $Mg^{++}$ (0·003 or 0·004 M) and *HDP* (0·0003 M) present throughout; $Al^{+++}$ and other metals, 0·0002 M. The other metals were also tested at 0·00002 M, without revealing any further activity. Other metals tested, at 0·0002 and 0·00002 M, with completely negative results, were $Zn^{++}$, $Ca^{++}$, $Sr^{++}$, $Cu^{++}$, $Hg^{++}$, $Ni^{++}$, $VO^{++}$, $Bi^{+++}$, $Ag^{+}$, and $Cd^{++}$. The salts used were the sulphates, except those of Be, Ca, Sr, Ba, Hg, Zr, Cs and Sn (chlorides), $UO_2$, La and Ag (nitrates), and Pb (acetate).)

| Addition | Glucose-1-phosphate converted in 15 min. (mg. P/100 ml.) 1st series | 2nd series | 3rd series | 4th series |
|---|---|---|---|---|
| None | 0·7 | 3·5 | 2·8 | 2·3 |
| $Al^{+++}$ | 16·2 | 21·7 | 12·3 | 25·6 |
| $Mn^{++}$ | 1·8 | — | — | — |
| $Be^{++}$ | 13·7 | — | — | — |
| $Cr^{+++}$ | 37·6 | — | — | — |
| $Fe^{+++}$ | 24·4 | — | — | — |
| $Ba^{++}$ | — | 8·4 | — | — |
| $Pb^{++}$ | — | 19·0 | — | — |
| $UO_2^{++}$ | — | 16·9 | — | — |
| $La^{+++}$ | — | — | 5·8 | — |
| $Zr^{++++}$ | — | — | 5·3 | — |
| $Ti^{++++}$ | — | — | 9·8 | — |
| $Ce^{+++}$ | — | — | 10·6 | — |
| $Cs^{+}$ | — | — | — | 4·0 |
| $Tl^{+}$ | — | — | — | 2·9 |
| $Sn^{++}$ | — | — | — | 5·8 |

With all these metals the activity was found only if $Mg^{++}$ was present at the same time (Table 4). A partial exception to this rule was found in $Cr^{+++}$, which at relatively high concentrations (of the order of $10^{-4}$ M) showed some activity in the absence of $Mg^{++}$ (see bottom of Table 4). This point will be considered in rather more detail later.

The relationship between the concentration of the individual metals and the degree of activity produced in the presence of $Mg^{++}$ and *HDP* showed in every case (except that of $Cr^{+++}$) a fairly sharp optimum. Although each single enzyme preparation gave a smooth and reproducible relationship between activity and concentration of cation, different preparations gave slightly different curves, so it is impossible to be very precise about the affinities of the enzyme for the metals. These points are illus-

Table 4. *The indispensability of* $Mg^{++}$

(Reaction mixture and conditions of experiment as before. *HDP* (0·0003 M) was present throughout; $Mg^{++}$, 0·003 M.)

| 'Second metal' | Concentration of 'second metal' (M × $10^5$) | Glucose-1-phosphate converted in 15 min. (mg. P/100 ml.) | | |
|---|---|---|---|---|
| | | $Mg^{++}$ alone | 'Second metal' alone | Both together |
| $Al^{+++}$ | 60 | 1·1 | 3·1 | 27·7 |
| $Al^{+++}$ | 40 | 0·7 | 0·4 | 23·4 |
| $Be^{++}$ | 23 | 0·8 | 0·0 | 9·8 |
| $UO_2^{++}$ | 67 | 0·0 | 0·0 | 9·6 |
| $UO_2^{++}$ | 20 | 1·5 | 1·1 | 9·5 |
| $Pb^{++}$ | 50 | 0·0 | 0·4 | 20·1 |
| $Fe^{+++}$ | 67 | 0·8 | 0·0 | 12·7 |
| $Ce^{+++}$ | 40 | 0·0 | 0·0 | 15·8 |
| $Cr^{+++}$ | 4 | 0·0 | 0·0 | 24·8 |
| $Cr^{+++}$ | 2 | 1·5 | 2·0 | 20·1 |
| $Cr^{+++}$ | 1·2 | 0·5 | 0·0 | 19·7 |
| $Cr^{+++}$ | 45 | 0·8 | 9·0 | 25·9 |
| $Cr^{+++}$ | 12 | 0·5 | 15·9 | 22·3 |

trated in the case of $Al^{+++}$ in Fig. 3; the other metals (except $Cr^{+++}$) gave curves of similar shape, whose characteristics are shown numerically in Table 5.

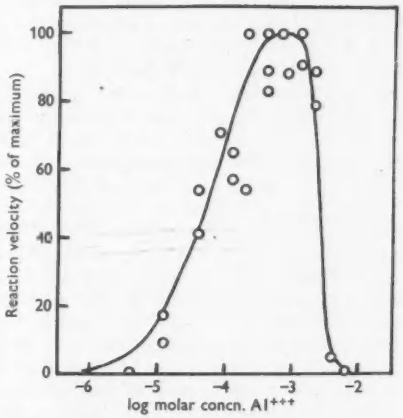


Fig. 3. The relationship between concentration of $Al^{+++}$ and activity of phosphoglucomutase. *HDP* (0·0003 M) and $Mg^{++}$ (0·003 M) present throughout.

The maximal activity reached with $Al^{+++}+Mg^{++}$, $Cr^{+++}+Mg^{++}$, and $Pb^{++}+Mg^{++}$, each at their optimal concentrations, was the same. With $Fe^{+++}$, $Fe^{++}$, and $Ce^{+++}$ the maximal activity in the presence of $Mg^{++}$ was only slightly lower, being from 70 to 90% of that with $Al^{+++}+Mg^{++}$. The other metals gave results which varied widely from one phosphoglucomutase preparation to another (Table 5). In view of this variability it must be admitted that some of the metals, which in Table 3 showed a small activity (e.g. $Ba^{++}$, $La^{+++}$, $Zr^{++++}$, and $Sn^{++}$) and which were there tested only with a single phosphoglucomutase preparation, might show greater activity if tests were carried out on a wider scale.

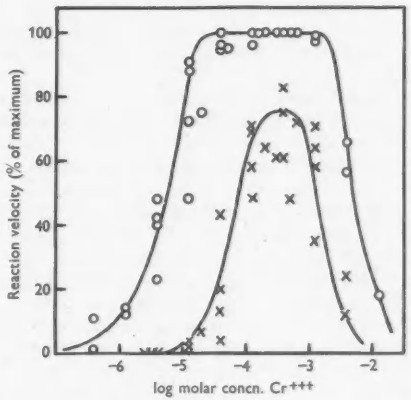


Fig. 4. The relationship between concentration of $Cr^{+++}$ and activity of phosphoglucomutase. Circles: in the presence of *HDP* (0·0003 M) and $Mg^{++}$ (0·003 M); crosses: in the presence of *HDP* (0·0003 M) only.

Chromium behaved exceptionally in several ways. In the presence of $Mg^{++}$, full activity equal to that with $Al^{+++}+Mg^{++}$ was found over the whole range of $Cr^{++}$ concentrations from $10^{-5}$ to $2\times10^{-3}$ M, and half the maximum was reached at $5\times10^{-6}$ M (Fig. 4). This high affinity of the enzyme for $Cr^{+++}$ distinguishes this element from $Al^{+++}$ and all the other metals. A second point of difference was that $Cr^{+++}$ showed a considerable degree of activity in the absence of $Mg^{++}$. This activity was only some 75% of that observed when $Mg^{++}$ was present too, and was found only at the higher part of the range

Table 5. *The relationships between the concentrations of various metals and the phosphoglucomutase activity produced by them in the presence of* $Mg^{++}+HDP$

| Metal | Salt used | Relative activity ($Al^{+++}=100$) | Concentration giving half the maximal activity (M) | Optimal concentration (M) |
|---|---|---|---|---|
| $Al^{+++}$ | $KAl(SO_4)_2.12H_2O$ | 100 | $6\times10^{-5}$ | $5\times10^{-4}$ |
| $Cr^{+++}$ | $KCr(SO_4)_2.12H_2O$ | 100 | $5\times10^{-6}$ | $10^{-5}$ to $2\times10^{-3}$ |
| $Pb^{++}$ | $Pb(OOC.CH_3)_2$ | 100 | $3\times10^{-5}$ | $4\times10^{-4}$ |
| $Fe^{+++}$ | $KFe(SO_4)_2.12H_2O$ | 70–80 | $1\times10^{-4}$ | $4\times10^{-4}$ |
| | $Fe(NO_3)_3.9H_2O$ | | | |
| $Fe^{++}$ | $Fe(NH_4)_2(SO_4)_2.6H_2O$ | 70 | $5\times10^{-5}$ | $4\times10^{-4}$ |
| $Ce^{+++}$ | $Ce_2(SO_4)_3$ | 80–90 | $6\times10^{-5}$ | $3\times10^{-4}$ |
| $Ti^{++++}$ | $Ti(SO_4)_2$ | 30–80 (5 enzyme samples) 0 (1 enzyme sample) | $4\times10^{-5}$ | $3\times10^{-4}$ |
| $UO_2^{++}$ | $UO_2(NO_3)_2.6H_2O$ | 35–80 (6 enzyme samples) 0 (2 enzyme samples) | $4\times10^{-4}$ | $3\times10^{-4}$ |
| $Be^{++}$ | $BeCl_2.4H_2O$ | 15–70 | $1\times10^{-4}$ | $5\times10^{-4}$ |

of concentrations of $Cr^{+++}$. There was consequently an appreciable range of $Cr^{+++}$ concentrations over which no activity was seen unless both metals were present together (from $10^{-5}$ to $4\times10^{-5}$ M). The activity with $Cr^{+++}$ alone showed a sharp maximum at $3\times10^{-4}$ M, half this maximum being reached at about $8\times10^{-5}$ M.

*Manganese and cobalt.* Cori *et al.* (1938) showed that the activity of phosphoglucomutase is increased by $Mg^{++}$, $Mn^{++}$ and $Co^{++}$, the degree of activity produced being roughly the same for all three, and the concentration required for full activation being greater for $Mg^{++}$ than for the other two. They also observed that at lower enzyme concentrations the efficacy of $Mg^{++}$ became less than that of $Mn^{++}$ or $Co^{++}$. However, at the still lower enzyme concentrations necessitated by the greater activity of the systems dealt with in the present work, the activity with $Mg^{++}$ was negligibly small, or zero (see Tables 4 and 6–8). Under the same conditions the activity with $Mn^{++}$ added alone was considerably greater than that with $Mg^{++}$ alone, but still small compared with that given by $Mg^{++}+Cr^{+++}+HDP$. The ratio

$$\frac{\text{Activity with } Mg^{++}+Cr^{+++}+HDP}{\text{Activity with } Mn^{++}}$$

for eight different enzyme preparations is shown in Table 6; it varied from 5 to 12, with an average of 7·5. $Co^{++}$, used alone, showed about the same activity as $Mn^{++}$ (Table 8).

In an earlier section it was stated that neither $Mn^{++}$ nor $Co^{++}$ could replace $Mg^{++}$ in the complete system (Table 2). In the experiments to which that table refers, these metals were tested at concentrations of 0·002 and 0·0002 M. The difference in behaviour was so curious that it seemed advisable to test these two metals more thoroughly, and in particular at the optimal concentrations already established by Cori *et al.* (1938). The results appear in Tables 6–8. It is plain that (*a*) neither $Mn^{++}$ nor $Co^{++}$ can act in the same way as $Mg^{++}$ in supplementing the action of $Cr^{+++}+HDP$, and (*b*) they will not supplement the action of any of the other metals which, in co-operation with $Mg^{++}+HDP$, can produce full activity of the enzyme. In fact, many of the metals which, when present with $Mg^{++}$, stimulate the enzyme, had an inhibitory effect on that activity which is produced by $Mn^{++}$ alone. Another point of interest is that the effect of $Mn^{++}$ is independent of the presence of *HDP* (see Table 9).

*The effect of* pH. The need for three activating components was observed equally at all pH values at which the enzyme was active (Table 10). These results were obtained with the use of a mixed phosphate (0·005 M) and veronal (0·005 M) buffer. Veronal at high concentrations inhibited the enzyme (50% inhibition at 0·0125 M), but at 0·005 M the inhibition was only *c.* 10%, and as the veronal was present in all the reaction mixtures the inhibition can be ignored. Borate is less suitable for buffering phosphoglucomutase, as it also inhibits it completely and at a rather lower concentration (50% inhibition at 0·007 M). The substrate also contributes largely to the buffering of the reaction mixtures used in these experiments, so the appropriate mixtures of glucose-1-phosphate, phosphate and veronal were made up, and their pH's adjusted to the required values by a colorimetric method, before they were measured out into the tubes in which the reaction was to take place. The fact that no difference in behaviour was noted obviated the need to make more precise measurements of the pH values. The enzyme preparation (at pH 7·5) was used at such great dilution that its addition had no effect on the pH of the mixtures.

Table 6. *The failure of* $Mn^{++}$ *to replace* $Mg^{++}$ *in the system* $Mg^{++}+Cr^{+++}+HDP$

(Reaction mixture and conditions of experiment as given in the text. Concentrations: *HDP*, 0·0003 M (present throughout); $Mg^{++}$, 0·003 M; $Mn^{++}$, 0·00125 M; $Cr^{+++}$, 0·00002 or 0·00004 M.)

| Glucose-1-phosphate converted in 15 min. (mg. P/100 ml.) in the presence of | | | | | Ratio of activities $\frac{Cr^{+++}+Mg^{++}}{Mn^{++}}$ |
|---|---|---|---|---|---|
| $Mg^{++}$ | $Mn^{++}$ | $Cr^{+++}$ | $Cr^{+++}+Mg^{++}$ | $Cr^{+++}+Mn^{++}$ | |
| 1·0 | 1·4 | 0·0 | 17·2 | 1·9 | 12·3 |
| 0·0 | 5·3 | 3·5 | 32·0 | 5·4 | 6·0 |
| 1·2 | 4·6 | 1·3 | 22·9 | 2·4 | 5·0 |
| 0·0 | 2·0 | 0·6 | 13·1 | 2·5 | 6·6 |
| 0·0 | 1·9 | — | 20·7 | — | 10·9 |
| 1·3 | 4·4 | — | 22·6 | — | 5·6 |
| 2·8 | 3·3 | — | 27·0 | — | 8·2 |
| 1·6 | 5·7 | 3·8 | 31·2 | — | 5·5 |

Table 7. *The failure of* $Mn^{++}$ *to replace* $Mg^{++}$ *in the system* $Mg^{++}+HDP+$ *other metals*

(Reaction mixtures and conditions of experiment as described in the text. Concentrations: *HDP*, 0·0003 M (present throughout); $Mg^{++}$, 0·003 M; $Mn^{++}$, 0·00125 M; $Al^{+++}$, 0·0004 M; $Pb^{++}$, 0·0005 M; $Fe^{+++}$, 0·00033 M; $UO_2^{++}$, 0·00067 M; $Ce^{+++}$, 0·0004 M.)

| Second metal ($X$) | Glucose-1-phosphate converted in 15 min. (mg. P/100 ml.) in the presence of | | | | |
|---|---|---|---|---|---|
| | $Mg^{++}$ | $Mn^{++}$ | $X$ | $Mg^{++}+X$ | $Mn^{++}+X$ |
| $Al^{+++}$ | 0·8 | 3·3 | 2·3 | 23·2 | 1·3 |
| $Al^{+++}$ | 1·2 | 4·6 | 4·6 | 18·8 | 0·7 |
| $Al^{+++}$ | 0·0 | 2·0 | 0·0 | 12·8 | 1·5 |
| $Fe^{+++}$ | 0·8 | 3·3 | 0·0 | 12·7 | 2·5 |
| $Fe^{+++}$ | 0·0 | 2·0 | 1·4 | 10·1 | 1·5 |
| $Pb^{++}$ | 0·0 | 2·3 | 0·4 | 10·1 | 0·0 |
| $UO_2^{++}$ | 0·0 | 2·3 | 0·0 | 9·6 | 0·0 |
| $Ce^{+++}$ | 0·0 | 2·3 | 0·0 | 15·8 | 1·6 |

Table 8. *The failure of* $Co^{++}$ *to replace* $Mg^{++}$ *in the system* $Mg^{++}+HDP+$ *various metals*

(Reaction mixture and conditions of experiment as before. Concentrations: *HDP*, 0·0003 M (present throughout); $Cr^{+++}$, 0·00002 or 0·00004 M; $Al^{+++}$, 0·0004 M; $Fe^{+++}$, 0·00033 M; $Pb^{++}$, 0·0005 M; $Ce^{+++}$, 0·0004 M; $Mg^{++}$, 0·003 M; $Co^{++}$, 0·0017 M.)

| Second metal ($X$) | Glucose-1-phosphate converted in 15 min. (mg. P/100 ml.) in the presence of | | | | |
|---|---|---|---|---|---|
| | $Mg^{++}$ | $Co^{++}$ | $X$ | $Mg^{++}+X$ | $Co^{++}+X$ |
| $Cr^{+++}$ | 1·2 | 5·7 | 1·3 | 22·9 | 7·1 |
| $Cr^{+++}$ | 0·0 | 1·9 | 0·6 | 13·1 | 2·5 |
| $Al^{+++}$ | 1·2 | 5·7 | 4·6 | 18·8 | 1·3 |
| $Al^{+++}$ | 0·0 | 1·9 | 0·0 | 12·8 | 1·1 |
| $Fe^{+++}$ | 0·0 | 1·9 | 1·4 | 10·1 | 0·3 |
| $Pb^{++}$ | 1·2 | 5·5 | 0·4 | 23·0 | 6·7 |
| $Ce^{+++}$ | 1·2 | 5·5 | 0·0 | 23·6 | 4·6 |

Table 9. *The absence of any effect of HDP on the activity of phosphoglucomutase activated by* $Mn^{++}$ *alone*

(Reaction mixture and conditions of experiment as before. Concentrations: $Mn^{++}$, 0·00125 M; *HDP*, 0·0003 M.)

| Glucose-1-phosphate converted in 15 min. (mg. P/100 ml.) | |
|---|---|
| *HDP* absent | *HDP* present |
| 24·0 | 24·4 |
| 12·0 | 11·0 |
| 6·0 | 6·5 |
| 3·1 | 2·6 |

*The effect of progressive dilution of the enzyme.* Since the activity of phosphoglucomutase with $Mg^{++}$, $Cr^{+++}$ and *HDP* is some ten times that previously observed with $Mg^{++}$ or $Mn^{++}$, it is clear that in order to measure this activity either the time scale of the experiments must be shortened or the enzyme concentration must be reduced. In the present work the method chosen was that of decreasing the enzyme concentration, keeping the time scale constant.

With the object of determining the proper enzyme concentration, a large number of routine tests have been carried out, using serial twofold dilutions of the enzyme, and the results of these show some

features of interest. Fig. 5 gives a diagrammatic summary of many such experiments. At the highest concentrations no activator was needed; with decreasing concentrations this activity rapidly fell off (Fig. 5, curve *A*), but full activity could still be achieved by addition of $Mg^{++}$ or $Mn^{++}$. With a small further dilution of the enzyme, the $Mg^{++}$ activation rapidly disappeared (curve *B*), while the activation with $Mn^{++}$ fell off more slowly (curve *C*). At a point where the activity with $Mg^{++}$ had vanished, and that with $Mn^{++}$ had become very small, full activity could still be produced by the addition of $Cr^{+++}$ and $Mg^{++}$. Eventually this activity also abruptly disappeared with further dilution (curve *D*).

Table 10. *The indispensability of two metals at various* pH *values*

(The preparation of the reaction mixtures and the conditions of experiment are described in the text. Concentrations: *HDP*, 0·0003 M (present throughout); $Cr^{+++}$, 0·00002 M; $Mg^{++}$, 0·003 M.)

| | Glucose-1-phosphate converted in 15 min. (mg. P/100 ml.) | | |
|---|---|---|---|
| | pH 7·5 | pH 8·0 | pH 8·5 |
| $Cr^{+++}$ alone | 1·2 | 2·8 | 1·4 |
| $Mg^{++}$ alone | 1·9 | 2·8 | 0·9 |
| $Cr^{+++} + Mg^{++}$ | 27·2 | 25·7 | 18·7 |

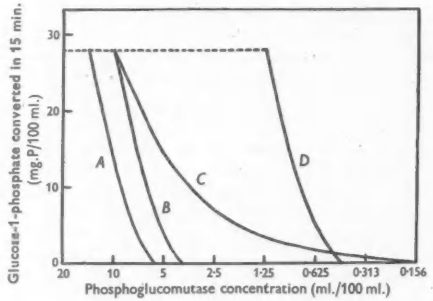


Fig. 5. Diagrammatic representation of the effect of dilution of phosphoglucomutase on its activity in the presence of various activators. Curve *A*, no activator; curve *B*, $Mg^{++}$; curve *C*, $Mn^{++}$; curve *D*, $Mg^{++} + Cr^{+++}$. *HDP* (0·0003 M) present throughout.

A curious point is that the velocity of reaction was proportional to the enzyme concentration only in the experiments where $Mn^{++}$ was the sole activator (Fig. 5, curve *C*; see also Table 9, where the four results were obtained with successive twofold dilutions of the enzyme). That the reaction velocity should diminish more rapidly than the enzyme concentration might be expected in experiments with no added activator, or in those with $Mg^{++}$ added alone (curves *A* and *B*), but in fact precisely the same relationship was observed when the enzyme was fully activated with $Mg^{++}$ and $Cr^{+++}$ (curve *D*).

Owing chiefly to variable losses in activity during the heat treatment in the purification of the enzyme, the absolute volume of enzyme required varied considerably from one preparation to another. On the other hand, the relative volumes required for measuring the activity in the presence of different combinations of activators were fairly constant. The scale of enzyme concentrations shown in Fig. 5 is the rough average of those found with a large number of phosphoglucomutase preparations, in which the final volume of the enzyme solution was one fifth of that of the original muscle extract.

*The absolute activity of phosphoglucomutase preparations.* Schlamowit & Greenberg (1948) say that the method of Colowick & Sutherland (1942) gives activities of phosphoglucomutase up to 3300 units/mg. of protein, these activities being measured in the presence of 0·00125 M $Mn^{++}$. Similar measurements on four samples of phosphoglucomutase in the presence of optimal concentrations of $Mg^{++}$, $Cr^{+++}$, and *HDP* gave values of 20,000, 37,000, 26,000 and 11,000 units/mg. dry weight, figures which confirm the value for the ratio:

$$\frac{\text{Activity with } Mg^{++} + Cr^{+++} + HDP}{\text{Activity with } Mn^{++}}$$

shown in Table 6.

## DISCUSSION

The chief facts presented in this communication do not call for further discussion at the moment. It might, however, be profitable to inquire into the possible physiological implications. Previously the most active form of phosphoglucomutase had been that obtained by the addition of $Co^{++}$, $Mn^{++}$ or $Mg^{++}$. The concentrations of $Co^{++}$ or $Mn^{++}$ required were enormously in excess of those which could occur in animal tissues, so $Mg^{++}$ was accepted as the only physiological activator. The present results show that, at a great dilution of the enzyme, $Mg^{++}$, either alone or in co-operation with *HDP*, imparts no activity to the enzyme, and that another metal is required in addition. This phenomenon is capable of more than one interpretation, but assuming for the moment that the activation by two metals is of importance *in vivo*, then only $Cr^{+++}$ appears to be worth considering as an actual physiological component of the system, because of the higher concentrations required with all the others. $Cr^{+++}$ shows maximal activity at a concentration of $10^{-5}$ M, or 50 $\mu$g./100 g. It remains to be seen whether a concentration of this order is to be found in animal tissues; the only report in the literature is that of Dutoit & Zbinden (1930), who by spectro-

graphic analysis detected traces of $Cr^{+++}$ in all organs, with most in thyroid and spleen.

The relationship between the activation by $Mn^{++}$ and that by $Mg^{++}+Cr^{+++}+HDP$ is curious. The variability in the ratio of these two activities (see Table 6) at first indicated that two different enzymes might be concerned, but the absence of any marked change in the ratio during the purification of the enzyme, and the variability in some other properties of the enzyme between one preparation and the next, suggest that this is not so. Further work on the interrelations between the activations by various metals and combinations is proceeding.

## SUMMARY

1. The greatest activity of phosphoglucomutase is found to occur only in the simultaneous presence of three activators, hexosediphosphate, $Mg^{++}$ and a second metal.

2. The second metal may be $Al^{+++}$, $Cr^{+++}$, $Pb^{++}$, $Fe^{+++}$, or $Ce^{+++}$; some other metals also show smaller activity.

3. In this system $Mg^{++}$ cannot be replaced by $Mn^{++}$ or $Co^{++}$. The maximal activity is some ten times that previously observed with $Mn^{++}$ as the only activator.

4. Of all the 'second metals' studied, only $Cr^{+++}$ shows any activity in the absence of $Mg^{++}$, and that only at relatively high concentrations.

5. Consideration of affinities suggests that if this 'two-metal' activation has any physiological importance, then $Cr^{+++}$ and $Mg^{++}$ are the metals concerned.

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# The Effect of the Peroxide Concentration and other Factors on the Decomposition of Hydrogen Peroxide by Catalase

By P. GEORGE,* *Molteno Institute, University of Cambridge*

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When catalase is added to hydrogen peroxide there is an initial rapid evolution of oxygen which lasts for about 2 min. After this oxygen is given off at a steady rate which slowly decreases in the course of about an hour. This is not necessarily due to a decrease in the peroxide concentration, since it is quite marked in experiments where there is a large excess of peroxide (Morgulis, Beber & Rabkin, 1926; George, 1947). The first problem in studying the kinetics of the reaction is to determine to what extent destruction of the enzyme is responsible for these changes in the rate as the reaction proceeds.

The results of some of the early investigations are difficult to interpret because only the total amount of oxygen evolved from a given amount of catalase and peroxide is recorded, so that the initial rapid reaction and the steady evolution cannot be distinguished. Provided the catalase is not present in excess the initial rapid reaction represents a small proportion of the total reaction possible, and as this condition obtained in most of the early investigations the results refer mainly to the subsequent steady rate. This is found to be directly proportional to the enzyme concentration, whereas the variation with peroxide concentration is more complicated. Above an optimum concentration as the peroxide is increased the reaction proceeds more slowly (Evans, 1907; Morgulis *et al.* 1926).

There is no doubt that the gradual decrease in the rate, after the initial rapid reaction is over, is due to enzyme destruction, and several kinetic equations have been developed to account for it in the papers of Yamasaki (1920), Morgulis (1921), Northrop (1924–5) and Williams (1927–8). There

* Present address: Department of Physical and Inorganic Chemistry, The University, Leeds 2.

remain two problems. Enzyme destruction may be one factor determining the variation of the steady rate with peroxide concentration. Partial enzyme destruction may be responsible for the transition from the initial rapid rate to the slower steady rate—the transition from α- to β-activity which was examined in a previous paper (George, 1947).

Experiments have been carried out to establish the variation of the reaction rate with enzyme and peroxide concentration by measuring the rates in the early stages of the reaction for different initial concentrations. With this method it is possible to determine the true kinetics of the $H_2O_2$ decomposition unaffected by the slow destruction of the enzyme, which makes the kinetic analysis of $H_2O_2$ concentration changes during a prolonged experiment extremely difficult. In the second part of the paper experiments are described which show that enzyme destruction is not responsible for either the kinetics of the steady reaction or the transition from α- to β-activity.

## EXPERIMENTAL

### *Materials and methods*

*Enzyme preparations.* A purified preparation of liver catalase (CL1) was obtained from horse liver by the method described by Keilin & Hartree (1945). Erythrocyte catalase was prepared from 2 l. fresh defibrinated horse blood, the red corpuscles being washed and plasmolyzed, and the haemoglobin removed by the Tsuchihashi method as described by Keilin & Mann (1940) in their preparation of carbonic anhydrase. Catalase was extracted from the resulting clear yellowish fluid by the process used for liver catalase, i.e. adsorption on calcium triphosphate gel followed by elution and fractional precipitation with $(NH_4)_2SO_4$; this was done twice, $NH_4OH$ being used for the first elution and $Na_2HPO_4$ for the second. Two specimens of erythrocyte catalase, CE1 and CE2 were obtained; CE1 was the purer sample, CE2 being extracted from the residues of the second adsorption. These specimens of catalase were analyzed for Fe, determined colorimetrically with 2:2′-dipyridyl, and for haemin, determined spectroscopically as pyridine haemochromogen (Keilin & Hartree, 1936), and compare favourably as regards purity with those used by Keilin & Hartree (1936) (Table 1). The low values for haemin are probably due to partial removal of the prosthetic group from the enzyme in the final stages of the purification. Two other specimens of liver catalase, CL2 and CL3, kindly provided by Dr E. F. Hartree, have also been used. Dilute catalase solutions were made each day from the sterile stock solutions (see Table 1), and stored in ice and water in a thermos flask. Some dilutions were made in 0·02% gelatin which had no effect on the rate of the reaction, but reduced inactivation of the enzyme during storage.

Table 1. *Analysis of catalase specimens*

(CL1 liver catalase, CE1 and CE2 erythrocyte catalase.)

| Analyses | Specimens CL1 | CE1 | CE2 |
|---|---|---|---|
| Dry weight (mg./ml.) | 34·1 | 5·2 | 5·4 |
| Haemin (% on dry wt.) | 0·51 | 0·47 | 0·50 |
| Total iron (% on dry wt.) | 0·14 | 0·11 | 0·09 |
| Haemin iron (as % of total iron) | 31 | 41 | 48 |

Keilin & Hartree (1936) found for three specimens 0·52, 0·55 and 0·37% haemin by weight.

A plasmolyzed red cell preparation used in later experiments was made by taking 1·0 ml. of a red cell suspension from defibrinated horse blood, which had been washed twice with 0·9% NaCl and spun down, and diluting to 250 ml. with distilled water.

*Procedure.* The $O_2$ evolution was measured as previously described in Barcroft manometers at 20° and in the pressure-gauge apparatus at 0° and 19·3–19·5° using the 'boat technique' of Meldrum & Roughton (1934) and George (1947). For the experiments in which dilute buffer solution was added to the reaction mixture during a run, flasks with a side tube of 1·5 ml. capacity were used. In the experiments where $H_2O_2$ was added during a run, two dangling tubes were used; the first with a short platinum hook contained dilute catalase solution and the second with a long platinum hook contained the required amount of '100 vol.' $H_2O_2$. Control experiments with no catalase present showed the blank reaction to be negligible in the concentration range 0–1·0 M-$H_2O_2$ where '20 vol.' $H_2O_2$ was used. For the range 1·0–5·0 M-$H_2O_2$, '100 vol.' $H_2O_2$ was used, the contribution of the blank being determined at each concentration, and subtracted from the experimental value. Before use the '100 vol.' $H_2O_2$ was brought to about pH 6·0 by adding a few drops of NaOH, bromocresol purple being used as indicator. In the majority of the experiments A.R. $H_2O_2$ was employed, but in one series a freshly prepared sample was used.

In all the experiments the total volume of $O_2$ evolved was very small compared with the $H_2O_2$ concentration which can be assumed to remain constant throughout each experiment. Protocols for the two methods of measuring gas evolution in experiments in which the peroxide concentration was varied are given below. *Barcroft manometers*: left-hand flask, 3·30 ml. $H_2O$; right-hand flask, 0·30 ml. 0·125 M-phosphate buffer, pH 5·85; 0·10 ml. 2% gelatin solution; 0·10 ml. dilute catalase solution in dangling tube; $H_2O$ and $H_2O_2$ to 3·30 ml. to give required molarity. *Boats*: Side '*a*': 1·0 ml. $H_2O$; 0·5 ml. dilute catalase solution; 0·5 ml. 0·2 M-buffer solution; side '*b*': 2·0 ml. $H_2O$ and $H_2O_2$ to give required molarity.

*Buffers.* To determine the effect of different buffer solutions of the same pH, Sorensen's citrate buffer and Clark & Lubs's phthalate buffer were used as well as Sorensen's phosphate buffer. The solutions were prepared as described by Clark (1925); the pH values, determined electrometrically, were 5·86, 5·84 and 5·85 at 20° respectively, the apparatus having been standardized with 0·05 M-potassium hydrogen phthalate solution, pH = 3·97 at 20°.

## RESULTS

### *Variation of the oxygen evolution rate with enzyme concentrations*

Barcroft manometers and the catalase specimen CL 2 were used at 20°, the peroxide concentration being kept at a constant value and the enzyme con-

centration varied. For 0·01, 0·03 and 0·5 M-$H_2O_2$ a dilute catalase solution was employed and between 0·02 and 0·30 ml. placed in the dangling tube; for 4·8 M-$H_2O_2$ a solution 10 times as concentrated was used. The results are given in Table 2, and show that for each peroxide concentration the rate is directly proportional to the catalase concentration.

Table 2. *Variation of the oxygen-evolution rate with enzyme concentration*

(Barcroft manometers at 20°; catalase CL2 and 0·3 ml. 0·125 M-phosphate buffer (pH 5·85) present in each experiment. 10 Enzyme units (e.u.) equivalent to $1·84 \times 10^{-4}$ mg. CL2.)

| $H_2O_2$ (M) | E.u. | Rate at times after start (μl. $O_2$/min.) | Rate/e.u. |
|---|---|---|---|
| | | 0–1 min. | |
| 0·01 | 5 | 20 | 4·0 |
| | 10 | 42 | 4·2 |
| | 15 | 58 | 3·9 |
| | 20 | 73 | 3·7 |
| | 25 | 90 | 3·6 |
| | 30 | 111 | 3·7 |
| | | 2–3 min. | |
| 0·03 | 2 | 14 | 7·0 |
| | 4 | 31 | 7·7 |
| | 6 | 43 | 7·2 |
| | 8 | 56 | 7·0 |
| | 10 | 68 | 6·8 |
| | | 2–3 min. | |
| 0·5 | 5 | 21 | 4·3 |
| | 10 | 42 | 4·2 |
| | 15 | 64 | 4·3 |
| | 20 | 82 | 4·1 |
| | 25 | 109 | 4·4 |
| | | 2–3 min. | |
| 4·8 | 10 | 7·5 | 0·75 |
| | 30 | 19·0 | 0·63 |
| | 50 | 29 | 0·58 |
| | 70 | 42 | 0·60 |
| | 90 | 58 | 0·64 |

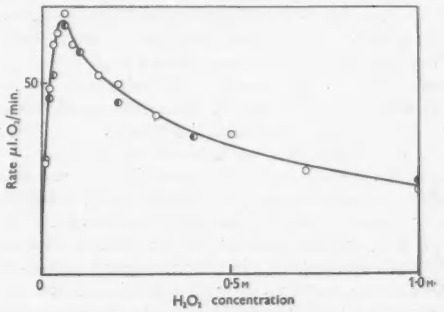


Fig. 1. Variation of the $O_2$-evolution rate, between the second and third minute from the start of the reaction, with the peroxide concentration. Measured with Barcroft differential manometers at 20° in the presence of 0·3 ml. 0·125 M-phosphate buffer, pH 5·85; ○—○, with liver catalase specimen CL1, $1·84 \times 10^{-4}$ mg.; ◐—◐, with erythrocyte catalase specimen CE1, $1·66 \times 10^{-4}$ mg.

*Variation of the oxygen-evolution rate by liver or erythrocyte catalase with peroxide concentration*

Barcroft manometers were used at 20°, the enzyme concentration being kept constant and the rate of $O_2$ evolution with different initial concentrations of peroxide determined. The $O_2$ evolved was measured for 10 min. at 0·5 and 1 min. intervals, and from the figures obtained the rate over any given interval could be calculated. Fig. 1 shows the rates for liver

Table 3. *Variation of the oxygen-evolution rate with peroxide concentration*

(Barcroft differential manometers at 20°; $1·84 \times 10^{-4}$ mg. catalase CL2 and 0·3 ml. 0·125 M-phosphate buffer (pH 5·85) present in each experiment.)

| $H_2O_2$ (M) | Rate at different times after beginning of reaction (μl. $O_2$/min.) | | | | |
|---|---|---|---|---|---|
| | 0–0·5 min. | 0·5–1 min. | 1–2 min. | 2–3 min. | 4–5 min. |
| 0·01 | 38 | 44 | 38 | 30 | 21 |
| 0·02 | 62 | 96 | 68 | 51 | 37 |
| 0·03 | 108 | 134 | 81 | 68 | 48 |
| 0·04 | 124 | 144 | 94 | 71 | 50 |
| 0·05 | 174 | 166 | 108 | 80 | 61 |
| 0·06 | 186 | 178 | 113 | 83* | 63 |
| 0·07 | 214 | 222 | 119* | 83* | 65* |
| 0·08 | 244 | 262* | 119* | 82 | 64 |
| 0·10 | 234 | 188 | 98 | 73 | 61 |
| 0·15 | 210 | 166 | 84 | 68 | 58 |
| 0·20 | 252 | 130 | 70 | 59 | 51 |
| 0·30 | 264 | 102 | 59 | 52 | 47 |
| 0·50 | 274* | 71 | 47 | 42 | 37 |
| 0·70 | 226 | 56 | 35 | 33 | 30 |
| 1·00 | 180 | 62 | 34 | 29 | 27 |
| 2·00 | 100 | 25 | 15·5 | 13·5 | 13·5 |
| 3·40 | 66 | 21 | 9·5 | 8·5 | 7·5 |
| 4·80 | 54 | 14 | 7·5 | 7·5 | 7·5 |

* Indicates maximum rate.

catalase CL 1 and erythrocyte catalase CL 1 over the range 0–1 M-$H_2O_2$, between the second and third minute from the start of the reaction, at which time most of the initial 'burst' is over (George, 1947). There is a very marked maximum in the rate at about 0·06 M-$H_2O_2$, and the catalase specimens from the two different sources give identical curves. The inhibition of the reaction at high $H_2O_2$ concentrations is very marked, for with 1·0, 2·0 and 4·0 M-$H_2O_2$ the rate is 32, 21 and 8 % respectively of the maximum rate at 0·06 M-$H_2O_2$.

No attempt has been made to compare the activities of the enzyme preparations on the basis of their haemin content. Catalase is an extremely active enzyme and the presence of about $1 \times 10^{-6}$ g. of inactive protein, and $1 \times 10^{-8}$ g. haemin or haemin degradation products is very unlikely to affect the rate of the reaction. For the kinetic analysis it is sufficient to show that with identical rates for the two specimens at, for instance, 1·0 M-$H_2O_2$, the same variation with $H_2O_2$ concentration is shown by each.

Similar results with liver catalase CL 2 at 20° are shown in Table 3 which shows the initial rapid evolution of $O_2$ followed by a lower steady rate which was discussed in a previous paper (George, 1947). The maximum initial rate, as given by the $O_2$ evolved during the first half minute of the reaction, occurs at a high peroxide concentration between 0·3 and 0·7 M-$H_2O_2$, whereas the maximum steady rate, measured by the $O_2$ evolved between the fourth and fifth minute from the start of the reaction, occurs at a much lower peroxide concentration—about 0·06–0·08 M-$H_2O_2$. The initial rates will be examined more fully in a later paper.

*Variation of the oxygen-evolution rate with peroxide or enzyme concentration using plasmolyzed cells*

To investigate the effect of impurities on the kinetics of the reaction, comparative experiments were carried out in the pressure gauge apparatus at 0° with erythrocyte catalase CE 2 ($1{\cdot}73 \times 10^{-3}$ mg.) and a suspension of plasmolyzed red blood cells (1/250 dilution, 0·2 ml.).

Fig. 2 shows that the same type of curve is given by the crude and the purified enzyme, and that the maximum occurs at the same $H_2O_2$ concentration. Table 4 shows that the rate is directly proportional to the concentration of lyzed cells. Since identical results are obtained with the purified enzyme extract and plasmolyzed red blood cells, the kinetics of the enzyme reaction are not affected by chance impurities in the enzyme preparation.

*Effect of different samples of hydrogen peroxide on the oxygen-evolution rate*

The inhibition of the reaction observed at high peroxide concentrations might be due to an inhibitory substance present in the specimen of $H_2O_2$

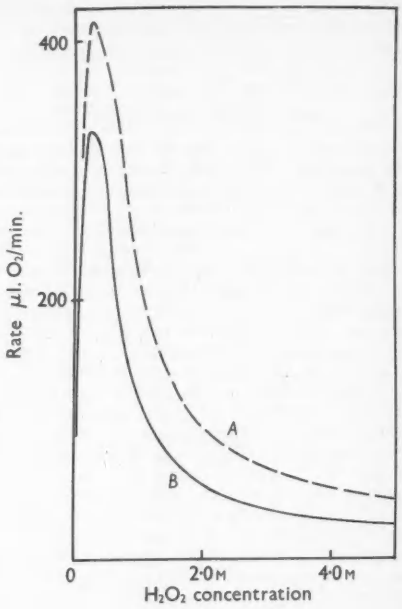


Fig. 2. A comparison of the variation of the $O_2$-evolution rate with peroxide concentration for a dilute suspension of lyzed red blood cells (curve *A*) and the purified erythrocyte catalase specimen CE 2 (curve *B*). Measurements of the rate in μl. $O_2$/min. made between 10 and 40 sec. from the start of the reaction with the pressure-gauge apparatus at 0°; 0·5 ml. 0·2 M-phosphate buffer (pH 5·85); $1{\cdot}73 \times 10^{-3}$ mg. catalase (CE 2) or 0·2 ml. 1/250 dilution lyzed cells present.

Table 4. *Variation of the oxygen-evolution rate with the concentration of plasmolyzed red blood cells*

(Pressure gauge apparatus at 0° in the presence of 0·5 ml. 0·2 M-phosphate buffer, pH 5·85. Lyzed cell concentration (L.C.) in ml. 1/250 dilution. Rate measured between 10 and 40 sec. from the start of the reaction.)

| $H_2O_2$ (M) | L.C. (ml.) | Rate (μl. $O_2$/min.) | Rate/ml. enzyme |
|---|---|---|---|
| 0·2 | 0·05 | 90 | 1800 |
| | 0·10 | 180 | 1800 |
| | 0·15 | 295 | 1980 |
| | 0·20 | 400 | 2000 |
| 1·0 | 0·1 | 85 | 850 |
| | 0·2 | 200 | 1000 |
| | 0·3 | 305 | 1020 |
| | 0·4 | 405 | 1010 |
| 5·0 | 0·2 | 40 | 200 |
| | 0·4 | 120 | 300 |
| | 0·6 | 180 | 300 |
| | 0·8 | 210 | 262 |

used. A sample of $H_2O_2$ was, therefore, prepared in the laboratory from $Na_2O_2$ and $H_2SO_4$ and purified by vacuum distillation (Kilpatrick, Reiff & Rice, 1926). Neither the standard sample nor the fresh sample showed any fluorescence when illuminated with strong ultraviolet light. Liver catalase CL 2 was used for the determinations which were carried out in Barcroft manometers at 20°. Table 5 shows

Table 5. *Comparison of the oxygen evolved from the standard peroxide and freshly prepared peroxide*

(Barcroft manometers at 20°; $1{\cdot}84 \times 10^{-4}$ mg. catalase CL 2 and 0·3 ml. 0·125 M-phosphate buffer (pH 5·85) present in each experiment. The average of five separate determinations given for each sample.)

| $H_2O_2$ (M) | Time after start (min.) | $O_2$ evolved (μl./min.) Standard $H_2O_2$ | Fresh $H_2O_2$ |
|---|---|---|---|
| 0·5 | 1–2 | 30·8 | 31·5 |
| | 2–3 | 29·8 | 29·6 |
| | 4–5 | 24·5 | 24·0 |
| 0·07 | 1–2 | 73·7 | 73·0 |
| | 2–3 | 52·5 | 51·4 |
| | 4–5 | 40·3 | 39·6 |

that there is a precise correspondence between the results for both specimens of $H_2O_2$, so the inhibition of the reaction at high $H_2O_2$ concentrations is not due to any inhibitory substance present in the peroxide.

*Effect of different buffer solutions of the same* pH *on the oxygen-evolution rate by catalase*

Anions are known to inhibit the decomposition of $H_2O_2$ by catalase owing to competition for the ferric iron of the haemin between $OH^-$ and the other anions (Agner & Theorell, 1946), which is more marked the greater the $H^+$-ion concentration. It was desirable to find out whether the inhibition observed at high $H_2O_2$ concentrations arises from a complex reaction involving the constituents of the buffer solution. Experiments were made using the pressure-gauge apparatus at 19·3–19·5° with liver catalase CL 3 to compare the effect of phosphate, citrate and phthalate buffers of pH values 5·85, 5·86 and 5·84 respectively, at concentrations between 0·005 and 0·1 M. The results are given in Table 6 for two concentrations of substrate (0·1 and 1·0 M-$H_2O_2$). The results show clearly that at pH 5·84–5·86 the rates are the same at 1·0 M-$H_2O_2$ and independent of the nature and concentration of the buffer solution used. At 0·1 M-$H_2O_2$ there may be a slight inhibition with the phthalate buffer, but the variation in the figures is of the same order as the experimental error. With all these buffers the steady rate with 0·1 M-$H_2O_2$ is between 2·0 and 2·4 times as great as the steady rate with 1·0 M-$H_2O_2$ as observed in the previous experiments, and there is no systematic fall in the rate as the buffer concentration is increased. This shows clearly that reactions of the anion or cation of the buffer solution do not play any part in determining the kinetics of the peroxide decomposition, and are not responsible for the inhibition of the decomposition observed at high $H_2O_2$ concentrations.

*Reversibility of the inhibition of catalase by high peroxide concentrations*

The question whether destruction of enzyme accounts for the reaction kinetics can be settled by experiments in which water or peroxide is added

Table 6. *Comparison of the oxygen-evolution rates with different buffers of the same* pH

(Pressure gauge apparatus at 19·3–19·5°; catalase CL 3, $7{\cdot}3 \times 10^{-4}$ mg.)

| Buffer Type | pH | Vol. (0·2 M-solution) | Rates (μl. $O_2$/min.) at time after start of reaction: 0·1 M-$H_2O_2$ 10–60 sec. | 0·1 M-$H_2O_2$ 2–4 min. | 1·0 M-$H_2O_2$ 10–60 sec. | 1·0 M-$H_2O_2$ 2–4 min. |
|---|---|---|---|---|---|---|
| Phosphate | 5·85 | 0·1 | 950 | 248 | 199 | 109 |
| | | 0·5 | 965 | 240 | 199 | 106 |
| | | 1·0 | 995 | 248 | 208 | 113 |
| | | 1·5 | 990 | 252 | 211 | 117 |
| | | 2·0 | 1040 | 235 | — | — |
| | | Av. | **988** | **245** | **204** | **111** |
| Citrate | 5·86 | 0·1 | 1060 | 232 | 204 | 113 |
| | | 0·5 | 988 | 226 | 198 | 109 |
| | | 1·0 | — | — | — | — |
| | | 1·5 | — | — | 228 | 114 |
| | | 2·0 | 960 | 218 | — | — |
| | | Av. | **996** | **225** | **210** | **112** |
| Phthalate | 5·84 | 0·1 | 955 | 223 | 202 | 102 |
| | | 0·5 | 955 | 218 | 199 | 100 |
| | | 2·0 | 950 | 215 | 230 | 103 |
| | | Av. | **953** | **219** | **210** | **102** |

during the reaction, for if complete reversibility is observed when the substrate concentration is brought to the optimum, then it is certain that enzyme destruction plays no part in determining the kinetics.

In the dilution experiments a buffer solution was added rather than water alone so that the ionic strength did not change, as an added safeguard that the observed effect is due only to the reaction between catalase and $H_2O_2$. The experiments were made in Barcroft manometers at 20°, a total of 1·5 ml. being used in each flask: the $O_2$ evolution was followed for 4·5 or 5 min., each experiment being done in triplicate. Catalase CL3 was employed, 0·1 ml. ($1·9 \times 10^{-5}$ mg.) being placed in the dangling tube: the concentration after mixing was about one fifth of that used in the previous experiments. With the usual amount of catalase the amount of $O_2$ evolved during the 5 min. of this experiment might amount to one quarter of the total $H_2O_2$ present, giving a substrate concentration below the optimum which would tend to mask the increase in the rate which is being looked for. Measurements of $O_2$ evolution were carried out at 0·01, 0·06, 0·36 and 1·46 M-$H_2O_2$, the appropriate blanks for the $H_2O_2$ alone being subtracted from the observed values. Two sets of experiments were made in which dilute buffer solution was added during the reaction, the flask with a side arm of capacity about 1·5 ml. being used. Typical protocols are given.

(*a*) *Concentration change from* 0·6 *to* 0·06 M-$H_2O_2$. Left-hand flask: 1·5 ml. $H_2O$. Right-hand flask: 0·094 ml. 1·92 N-$H_2O_2$, 0·06 ml. 0·2 M-phosphate buffer (pH 5·85), 0·10 ml. dilute catalase solution in dangling tube; total = 0·25 ml. 0·36 M-$H_2O_2$; side arm, 0·95 ml. $H_2O$, 0·30 ml. 0·2 M-phosphate buffer (pH 5·85); total 1·25 ml.; after mixing total volume = 1·50 ml. 0·06 M-$H_2O_2$. With this arrangement the evolution of $O_2$ can first be determined for 0·25 ml. of 0·36 M-$H_2O_2$, and then, by allowing the dilute buffer solution to run into the flask by rotating the side arm, measurements can be continued for 1·5 ml. of 0·06 M-$H_2O_2$; any alteration in the rate is to be attributed only to the change in $H_2O_2$ concentration. This was checked by an experiment where the $H_2O_2$ concentration was kept constant (Fig. 3, curve *A*), the $O_2$ evolution from 0·25 ml. of 0·36 M-$H_2O_2$ with 0·10 ml. dilute catalase being the same as that from 1·50 ml. of 0·36 M-$H_2O_2$ with 0·10 ml. dilute catalase, i.e. the same amount but one sixth the previous concentration. Curve *B* gives the $O_2$ evolution for 1·50 ml. of 0·06 M-$H_2O_2$ with 0·10 ml. dilute catalase. In experiments illustrated by curves *C* and *D* the reaction was started with 0·25 ml. of 0·36 M-$H_2O_2$ and after 2 and 1 min. respectively 1·25 ml. of dilute buffer solution were added according to the protocol above; in both cases the $O_2$ evolution after dilution increases and follows the points on curve *B*, the appropriate curve for the lower $H_2O_2$ concentration. The inhibition observed with 0·36 M-$H_2O_2$ can, therefore, be reversed by dilution during the reaction, the reversibility being quantitative to within experimental error. The rates of $O_2$ evolution, $\mu$l. $O_2$/min., measured over the period 2–4·5 min. from curves *A*, *B* and *C* are 7·0, 10·5 and 10·4 respectively. Curve *D*,

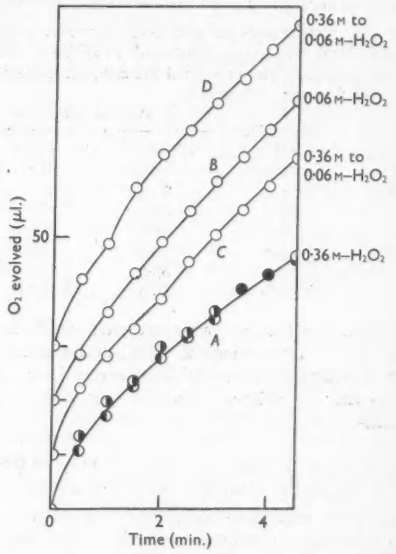


Fig. 3. $O_2$-evolution curves for 0·36 and 0·06 M-$H_2O_2$ with $1·9 \times 10^{-5}$ mg. of catalase (CL3) in each experiment. Curve *A*: 0·36 M-$H_2O_2$, 0·25 ml. solution points ◑; 1·50 ml. solution points ◐. Curve *B*: 0·06 M-$H_2O_2$, 1·50 ml. solution. Curve *C*: from 0 to 2 min. 0·25 ml. 0·36 M-$H_2O_2$, then 1·25 ml. dilute buffer solution added from a side arm giving 1·50 ml. 0·06 M-$H_2O_2$. Curve *D*: as for curve *C* but addition of dilute buffer solution at the end of the first minute. Measurements made with Barcroft differential manometers at 20° in phosphate buffer (pH 5·85), concentration in each experiment 0·024 M.

illustrating the effect of dilution at the end of the first minute of the reaction, will be discussed later when the possibility of enzyme destruction as the cause of the decay of the initial rapid reaction is examined.

(*b*) *Concentration change from* 0·06 *to* 0·01 M-$H_2O_2$. The results in Table 3 show too that dilution from 0·06 to 0·01 M-$H_2O_2$ should cause a marked decrease in the rate. This was tested in a similar way to the foregoing experiments, using 0·32 N-$H_2O_2$ in place of 1·92 N-$H_2O_2$. The average results for these separate

determinations of $O_2$ evolution with each $H_2O_2$ concentration are given in Fig. 4. Curves *A* and *B* show the $O_2$ evolution for 1·50 ml. of 0·01 M-$H_2O_2$ and 0·25 ml. of 0·06 M-$H_2O_2$, the rate being greater at the higher $H_2O_2$ concentration; curve *C* gives the $O_2$ evolution for the reaction starting with 0·25 ml. of 0·06 M-$H_2O_2$ and adding 1·25 ml. of buffer solution after 2 min., and shows the expected decrease in the rate. The rates of $O_2$ evolution, measured over the period 2–4·5 min. from curves *B*, *A* and *C* are 10·4, 4·8 and 4·4 $\mu$l. $O_2$/min. respectively.

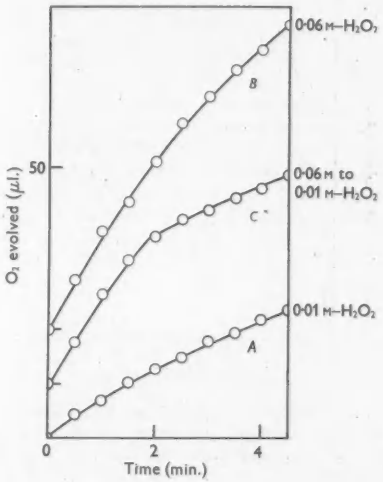


Fig. 4. $O_2$-evolution curves for 0·01 and 0·06 M-$H_2O_2$ with $1·9 \times 10^{-5}$ mg. catalase (CL3) in each experiment. Curve *A*: 0·01 M-$H_2O_2$, 1·50 ml. solution. Curve *B*: 0·06 M-$H_2O_2$, 0·25 ml. solution. Curve *C*: from 0 to 2 min. 0·25 ml. 0·06 M-$H_2O_2$, then 1·25 ml. dilute buffer solution added from a side arm giving 1·50 ml. 0·01 M-$H_2O_2$. Measurements made with Barcroft differential manometers at 20° in phosphate buffer (pH 5·85), concentration in each experiment 0·024 M.

(*c*) *Concentration change from* 0·01 *to* 0·36 M-$H_2O_2$. Two sets of experiments were then made in which a small amount of '100 vol.' $H_2O_2$ was added during the reaction from a second dangling tube with a long platinum hook. The dilute catalase solution was contained in the first dangling tube, which had a short platinum hook, and by careful tapping could be dislodged without upsetting the second tube holding the concentrated $H_2O_2$. *Protocol:* left-hand flask, 1·5 ml. $H_2O$; right-hand flask, 0·094 ml. 0·32 N-$H_2O_2$, 0·40 ml. 0·2 M-phosphate buffer (pH 5·85), 0·90 ml. $H_2O$, 0·10 ml. catalase (= 1·50 ml. 0·01 M-$H_2O_2$); 0·05 ml. 10·5 M-$H_2O_2$ in second dangling tube. Total volume = 1·50 ml. 0·36 M-$H_2O_2$. It is important to show that by increasing the $H_2O_2$ concentration the rate can also be increased, for this would confirm that the inhibition noted at high $H_2O_2$ concentrations is not caused by an irreversible oxidative degradation of the enzyme. This can be tested in the concentration range 0·01–0·36 M-$H_2O_2$: the direct determination of the rates at these two concentrations recorded in Table 3 shows about a twofold increase. Fig. 5 shows the average of three separate determinations of $O_2$ evolution at each of these $H_2O_2$ concentrations, curve *A*, 1·50 ml. of 0·01 M-$H_2O_2$, and curve *B*, 1·50 ml. of 0·36 M-$H_2O_2$. Curve *C* gives the $O_2$ evolution for the reaction

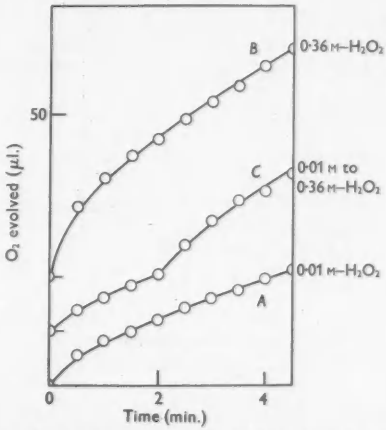


Fig. 5. $O_2$-evolution curves for 0·01 and 0·36 M-$H_2O_2$ with $1·9 \times 10^{-5}$ mg. catalase (CL3) in each experiment. Curve *A*: 0·01 M-$H_2O_2$, 1·50 ml. solution. Curve *B*: 0·36 M-$H_2O_2$, 1·50 ml. solution. Curve *C*: from 0 to 2 min. 1·50 ml. 0·01 M-$H_2O_2$, then 0·05 ml. 10·5 M-$H_2O_2$ added from a second dangling tube giving 1·55 ml. 0·36 M-$H_2O_2$. Measurements made with Barcroft differential manometers at 20° in phosphate buffer (pH 5·85), concentration in each experiment 0·024 M.

starting with 1·50 ml. of 0·01 M-$H_2O_2$ and adding 0·05 ml. of 10·5 M-$H_2O_2$ from the second dangling tube after 2 min. to give 0·36 M-$H_2O_2$. A marked increase in the rate is observed, which corresponds to that expected for the increase in $H_2O_2$ concentration. The rates of $O_2$ evolution measured over the period 2–4·5 min., from curves *A*, *B* and *C*, are 3·7, 7·0 and 7·4 $\mu$l. $O_2$/min. respectively.

(*d*) *Concentration change from* 0·06 *to* 1·46 M-$H_2O_2$. The inhibitory effect of strong $H_2O_2$ which was observed in the direct determinations of the rate at different $H_2O_2$ concentrations (Fig. 2 and Table 3) was confirmed by adding peroxide during the reaction to bring the concentration from 0·06 to 1·46 M-$H_2O_2$ when the rate should fall to about one third of its previous value. In Fig. 6, curves *A* and *B* refer to the $O_2$ evolution from 1·50 ml. of 1·46 and

0·06 M-$H_2O_2$ respectively, while curve *C* gives the $O_2$ evolved from 1·30 ml. of 0·06 M-$H_2O_2$ for 2 min., when 0·20 ml. of 10·5 M-$H_2O_2$ was added and readings taken for a further 3 min. The rate decreases quantitatively corresponding to the peroxide or the nature and concentration of the buffer solution used. The quantitative reversal of the inhibition shown by the addition of dilute buffer solution or peroxide during the reaction shows that the phenomenon is not due to destruction of the enzyme. The remaining problem to be settled is whether or not partial enzyme destruction is responsible for the transition from the initial rapid rate to the steady rate. The experiment recorded in Fig. 3, curve *C*, shows that it is not.

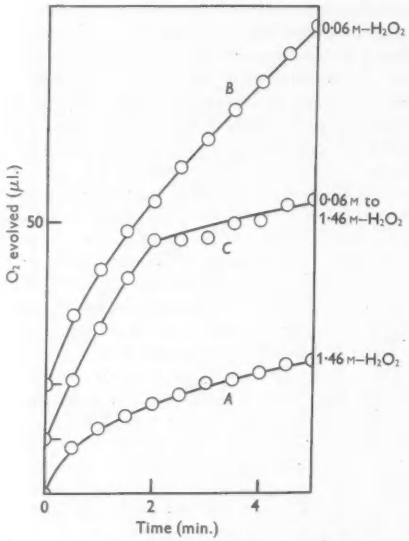


Fig. 6. $O_2$-evolution curves for 1·46 and 0·06 M-$H_2O_2$ with $1·9 \times 10^{-5}$ mg. catalase (CL3) in each experiment. Curve *A*: 1·46 M-$H_2O_2$, 1·50 ml. solution. Curve *B*: 0·06 M-$H_2O_2$, 1·50 ml. solution. Curve *C*: from 0 to 2 min. 1·30 ml. 0·06 M-$H_2O_2$, then 0·20 ml. 10·5 M-$H_2O_2$ added from a second dangling tube giving 1·50 ml. 1·46 M-$H_2O_2$. Measurements made with Barcroft differential manometers at 20° in phosphate buffer (pH 5·85), concentration in each experiment 0·024 M.

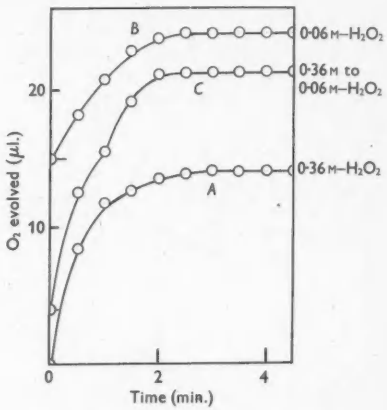


Fig. 7. $O_2$ evolved during the initial rapid reaction with 0·36 and 0·06 M-$H_2O_2$, calculated from the $O_2$-evolution curves in Fig. 3 by subtracting the $O_2$ evolved in the steady reaction. Curve *A* (from Fig. 3*A*): 0·36 M-$H_2O_2$, 0·25 ml. solution. Curve *B* (from Fig. 3*B*): 0·06 M-$H_2O_2$, 1·50 ml. solution. Curve *C* (from Fig. 3*D*): from 0 to 1 min. 0·25 ml. 0·36 M-$H_2O_2$, then 1·25 ml. dilute buffer solution added from a side arm giving 1·50 ml. 0·06 M-$H_2O_2$.

change in $H_2O_2$ concentration. The rates of $O_2$ evolution, measured over the period 2–4·5 min., from curves *B*, *A* and *C* are 10·9, 2·9 and 2·9 μl. $O_2$/min. respectively.

## DISCUSSION

The experiments described above show that the rate of decomposition of $H_2O_2$ by catalase is directly proportional to the enzyme concentration and shows a complex variation with the $H_2O_2$ concentration. Below 0·06 M-$H_2O_2$ the rate is directly proportional, between 0·06 and 0·08 M-$H_2O_2$ there is a maximum in the rate, above 0·08 M-$H_2O_2$ further increase in the peroxide decreases the rate. These results, particularly the inhibition at high $H_2O_2$ concentration, are in no way determined by the purity of the enzyme, the presence of inhibitors in the

The experiment is one of the series in which dilute buffer solution was added during the reaction, the addition being made at the end of the first minute, i.e. during the period of the rapid reaction. The concentration change was from 0·36 to 0·06 M-$H_2O_2$. The effect of this dilution during the initial rapid reaction can best be seen by subtracting from all the experimental points in Fig. 3 the appropriate contribution of the steady rate. This is done in Fig. 7, where curves *A*, *B* and *C* give the $O_2$ evolution for the calculated rapid reaction for 0·36, 0·06 and 0·36 → 0·06 M-$H_2O_2$, from the experimental points in Fig. 3, curves *A*, *B* and *D*. The rapid reaction is over more quickly the higher the peroxide concentration. With 0·36 M-$H_2O_2$, 83 % is over in the first minute, with 0·06 M-$H_2O_2$, 65 %. Curve *C*, Fig. 7, shows that after dilution a greater quantity of $O_2$ is evolved in completing the initial rapid reaction than is evolved in the more concentrated solution. This suggests

that the transition to the steady rate cannot be due to any partial destruction of the enzyme. If it were, no increase in $O_2$ evolution would be observed on dilution. The explanation for this transition from α- to β-activity in the decomposition of $H_2O_2$ by catalase should be sought in the series of unit reactions involved in the peroxide decomposition. In this respect there is a similarity in the kinetics of the enzymatic decomposition and of the reaction catalyzed by ferrous iron (George, 1947), even though the iron in catalase is in the ferric state.

## SUMMARY

1. The evolution of oxygen from hydrogen peroxide at pH 5·58 by liver catalase, erythrocyte catalase and a suspension of plasmolyzed red blood corpuscles has been measured manometrically, and by the 'boat technique' of Meldrum & Roughton (1934) using a pressure gauge for following rapid rates. There is an initial rapid evolution of $O_2$ followed by a slower steady evolution: a transition from α- to β-activity.

2. Identical kinetic relationships are obtained with the three specimens of catalase. The rate is directly proportional to the catalase concentration. The variation of the rate with peroxide concentration shows a maximum at about 0·07 M-$H_2O_2$, further increase in the $H_2O_2$ concentration decreasing the rate very markedly.

3. These kinetic relationships, particularly the inhibition at high $H_2O_2$ concentrations, are in no way determined by the purity of the enzyme, the presence of inhibitors in the peroxide or the nature and concentration of the buffer solution used.

4. The quantitative reversal of the inhibition shown by addition of dilute buffer solution or peroxide during the reaction shows that enzyme destruction plays no part in determining these kinetic relationships.

5. Dilution during the period of initial rapid activity shows a similar reversibility which suggests that the transition from α- to β-activity is not caused by the partial destruction of the enzyme.

I wish to thank Prof. D. Keilin, F.R.S., for his stimulating interest and kind encouragement in this work.

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# Activity of the Succinic Dehydrogenase-cytochrome System in Different Tissue Preparations

By D. KEILIN and E. F. HARTREE, *Molteno Institute, University of Cambridge*

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The object of this paper is the study of the effect of different factors on the rate of aerobic oxidation of succinic acid by various tissue preparations. From the results obtained it is apparent that there are broadly speaking two types of factor: first, those which may react directly and specifically with one or more components of the succinic dehydrogenase-cytochrome system or of other catalytic mechanisms linked to this system; and secondly those which, by modifying the colloidal state of the enzyme preparations, may markedly increase or decrease their activity. The significance of the latter type of factor has not hitherto been fully recognized, with the result that the mode of action of a number of inhibitors and activators on the succinic system in tissue extracts has been misinterpreted, and the activators have erroneously been considered as essential links in the catalytic chain which brings about the aerobic oxidation of succinate. This study will enable us to examine critically the claims of

previous workers to have discovered or even isolated such additional catalytic intermediates in the succinic system.

## MATERIALS AND METHODS

The preparation of heart muscle (from horse unless otherwise stated) and pigeon-breast-muscle extracts and of denatured globin (from recrystallized horse oxyhaemoglobin) have already been described (Keilin & Hartree, 1947*a*, *b*). If prepared exactly according to our description the heart preparation will yield, on 20-fold dilution with 0·1 M-phosphate (pH 7·3), a homogeneous opalescent fluid which shows no sign of aggregation on standing for several hours at room temperature. The appearance of a flocculent precipitate indicates either that the pH became too low (<5·5) during the isolation of the enzyme material, or that it was not kept cold at that stage. Although such materials may show catalytic activity, they are unsuitable for the experiments described in this paper. Cytochrome *c* was prepared from horse heart (Keilin & Hartree, 1945) and a 1% solution of the pigment, containing 0·34% Fe, was generally used. In certain experiments cytochrome *c* containing 0·43% Fe was used.

*Kidney oxidase preparations.* The cortical layer of 1 horse kidney was passed through a Latapie mincer, stirred 10 min. with 1 l. distilled water and pressed out in muslin. The pulp was stirred with a further 1 l. water and pressed out again. The combined fluids were centrifuged to remove solid particles and the cloudy fluid cooled to 0° was brought to pH 5·5 with N-acetic acid. The precipitate was immediately centrifuged down, washed by centrifuging with 0·02 M-$KH_2PO_4$, and the solid material resuspended in its own volume of 0·1 M-phosphate buffer (pH 7·3).

*Vacuum-dried preparations.* Heart-muscle preparation (20 ml.) in a 500 ml. beaker was evacuated to 0·1 mm. Hg in a desiccator over KOH and concentrated $H_2SO_4$. Within 10–20 min. the material froze as a result of the rapid evaporation. The desiccator was then detached from the pump and allowed to stand evacuated overnight. In this way a light flaky product was obtained, very similar in appearance to a freeze-dried protein.

*Frozen oxidase preparations.* Heart-muscle preparations were frozen three times, either in liquid air or in an ice-salt freezing mixture, with complete thawing after each freezing. This treatment resulted in an irreversible flocculation of the colloidal suspension.

*Glyoxaline buffer.* As veronal inhibits succinic dehydrogenase, phosphate-free buffers (pH 7·3) were made up from 4(5)-methylglyoxaline (Windaus & Knoop, 1905) according to the description of Kirby & Neuberger (1938).

*The oxidation of succinate* was followed in differential manometers at 39° with 3·3 ml. fluid in each flask as already described (Keilin & Hartree, 1947*b*). Unless otherwise stated, the concentration of buffer was 0·07 M with respect to phosphate or glyoxaline. The right-hand flasks received the appropriate oxidase preparation and 0·2 ml. 1% cytochrome *c*. Sodium succinate was added from a dangling tube, its concentration after mixing with the contents of the flask being 0·04 M. All experiments were carried out at pH 7·3. When frozen or dried preparations were tested this fact is stated; in all other cases the fresh material was used. The term $Q_{O_2}$ used in this paper corresponds to $\mu$l. $O_2$ taken up/mg. dry wt. of preparation/hr.

## EXPERIMENTAL

### A. *Effect of varying concentrations of phosphate and glyoxaline buffers*

Our experiments have shown that phosphate buffer cannot be considered as an indifferent medium for the study of the succinic dehydrogenase-cytochrome system.

Fig. 1, which summarizes the experimental results obtained with the heart-muscle preparation before and after vacuum drying, clearly indicates that the rate of oxidation of succinic acid increases with the decrease in phosphate concentration down to 0·15 M, but at lower concentrations the rate falls again. In the case of the undried material these changes in the activity of the preparation follow the

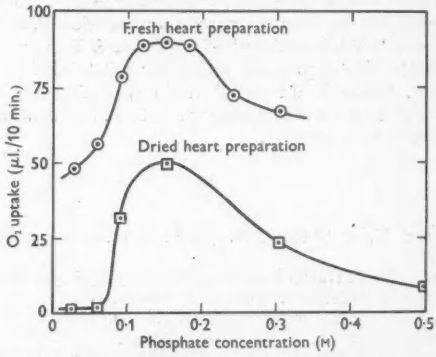



Fig. 1. Effect of phosphate buffer concentration on the rate of $O_2$ uptake of fresh and vacuum-dried heart preparations in presence of succinate and cytochrome *c*. Dry wt. of preparation = 1·1 mg.; temp., 39°; pH, 7·3.

degree of dispersion of the protein, the most complete dispersion being at 0·15 M-phosphate with a tendency towards flocculation as the concentration of phosphate is either decreased or increased. When phosphate is replaced by the glyoxaline buffer the rate of oxidation of succinate increases with the decrease of buffer concentration. For such experiments glyoxaline buffer must be used instead of phosphate in the preparation of the heart-muscle extract. The peculiar effect of phosphate can be illustrated from experiments where the total salt concentration (phosphate + chloride) remained constant while the phosphate concentration, denoted as [phosph], was varied. In such a case there is a definite activation by phosphate when comparison is made with control experiments in glyoxaline buffer (Table 1).* The combination of this activation by [phosph] and the general salt effect presumably gives rise to the maxima in Fig. 1. No optimal effect of [phosph] is shown by kidney preparations where the reaction rate falls off with increasing

* Throughout this paper we use the term 'activation' to indicate an increase in the rate of $O_2$ uptake of the entire catalytic system.

Table 1. *Comparison of the effects of phosphate and glyoxaline buffers on the oxidation of succinate by a heart oxidase preparation*

(Total volume of oxidase (0·05 ml.) + cytochrome *c* + succinate = 1·0 ml. NaCl and buffers: 0·1 M; temperature, 39°; pH, 7·3.)

| | $O_2$ uptake (μl./20 min.) | |
|---|---|---|
| | In phosphate | In glyoxaline |
| Oxid. + cyt. *c* + succ. + 1·3 ml. buffer + 1·0 ml. NaCl | 87 | 97 |
| Oxid. + cyt. *c* + succ. + 1·8 ml. buffer + 0·5 ml. NaCl | 116 | 100 |
| Oxid. + cyt. *c* + succ. + 2·3 ml. buffer + 0 ml. NaCl | 138 | 99 |

Table 2. *The effect of phosphate concentration on the oxidation of succinate by a kidney preparation, and the effect of cytochrome* c

(Kidney oxidase (0·25 ml.); succinate; temp., 39°; pH, 7·3.)

| Phosphate conc. (M) | μl. $O_2$ uptake in 10 min. | |
|---|---|---|
| | No cyt. *c* | With cyt. *c* |
| 0·015 | 20 | 96 |
| 0·06 | 14 | 77 |
| 0·15 | 8 | 18 |
| 0·30 | 1 | 9 |

[phosph] (Table 2). The rate of reduction of methylene blue in Thunberg vacuum tubes by succinate and the various preparations is independent of [phosph] over a wide range.

### B. *Effect of adenosinetriphosphate (ATP)*

Biró & Szent-Györgyi jun. (1946) tested the effect of ATP on the oxidation of succinic acid by a preparation obtained from rabbit-skeletal muscle minced and washed twice in 20 volumes of water. The maximum $Q_{O_2}$ of this muscle preparation at 37° without and with ATP was found by them to be 2·5 and 5 respectively. They concluded that 'the activity of the succinoxidase is greatly dependent on the presence of ATP'.

As the activity of their enzyme system was very low we considered it interesting to determine the effect of ATP on our enzyme preparations, the $Q_{O_2}$ of which varies between 500 and 700. The experiments, carried out in the usual way, showed that the $Q_{O_2}$ of a horse-heart preparation in phosphate buffer without and with 0·003 M-ATP (5 mg./manometer flask) was 620 and 540 respectively. In other words, ATP produced about 13% inhibition. Experiments carried out on a heart-muscle extract prepared and tested in glyoxaline buffer gave similar results: namely, ATP, in presence or in absence of K ions, invariably produced about 15% inhibition. The glyoxaline heart-muscle preparation was found to contain only a trace of inorganic phosphate and its adenosinetriphosphatase (ATP-ase) activity was very low. Thus 0·2 ml. of this preparation (i.e. 4 times the amount used in manometric experiments) contained 5 μg. inorganic P and when incubated with 5 mg. ATP at 39° for 50 min., hydrolyzed only 8% of the substrate.

Since the heart-muscle oxidase produced by our method contains very little ATP-ase further experiments were carried out on a pigeon-breast-muscle preparation (Keilin & Hartree, 1947*b*) using both phosphate and glyoxaline buffers for extraction. Several samples were collected in the course of fractionation of the muscle and these differed in their activities with respect to succinate, and in their contents of myosin and ATP-ase. An examination of these fractions in glyoxaline and in phosphate buffer showed that the addition of ATP produced inhibitions varying from ·25 to 37%.

We can say in conclusion that the results of our experiments, unlike those of Biró & Szent-Györgyi jun. (1946), show that ATP inhibits the catalytic activity of highly active preparations oxidizing succinic acid. The inhibitory effect of ATP is, in fact, of the same order as that produced by an equivalent concentration of inorganic pyrophosphate.

### C. *Effect of proteins on the activity of the succinic systems in different preparations*

The activation by denatured globin of a heart-muscle preparation oxidizing succinate was recorded in a previous paper (Keilin & Hartree, 1947*b*) under a single set of experimental conditions. Further work revealed that other proteins exhibited the same property, and that the magnitude of the effect was greatly dependent upon phosphate concentration. Such experiments showed that, except at very high [phosph], the activity of a heart preparation in presence of added proteins was independent of [phosph] and that the effect of protein in sub-optimal [phosph] was to raise the activity of the preparation to the optimal level (Table 3). From

Table 3. *The effect of phosphate concentration on the oxidation of succinate by a heart preparation ± added protein*

(Oxidase (0·04 ml.); succinate and cytochrome *c*; 0·3 ml. 1·4% denatured globin or 0·3 ml. horse plasma; temp., 39°; pH, 7·3.)

| Phosphate conc. (M) | $O_2$ uptake (μl./30 min.) | | |
|---|---|---|---|
| | Alone | + Plasma | + Globin |
| 0·03 | 198 | 320 | 349 |
| 0·09 | 294 | 348 | — |
| 0·15 | 325 | 340 | 331 |
| 0·30 | 279 | 279 | — |

this it follows that the effect of proteins can only be studied at low [phosph] and the concentration chosen for subsequent work was 0·07 M. It is important to note that denatured globin is completely precipitated at the pH of our experiments (7·3). From the results of experiments carried out

with an ox-heart preparation and summarized in Table 4 it is evident that proteins generally, but not their digests, activate the oxidation of succinate, and that denatured and consequently insoluble proteins are the most efficient in this respect. Similar results were obtained with a horse-heart preparation.

Table 4. *The effects of various substances on the aerobic oxidation of succinate by a pig-heart preparation*

(Phosphate (0·07 M); oxidase (0·05 ml.); succinate, cytochrome *c*; temp., 39°; pH, 7·3.)

| Substance added | Relative rate of $O_2$ uptake |
|---|---|
| None | 100 |
| Crystalline serum albumin (2%) | 219 |
| Peptone (2%) | 117 |
| Gelatine (2%) | 200 |
| Casein (commercial (2%)) | 171 |
| Tryptic digest of casein (2%) | 122 |
| Horse plasma (0·4 ml.) | 210 |
| Methaemoglobin (2%) | 206 |
| Denatured globin (2%) | 285* |
| Boiled methaemoglobin (2%) | 244* |
| $Ca_3(PO_4)_2$ gel (30 mg.) | 199 |
| $CaCl_2 + AlCl_3$ (each $4 \times 10^{-4}$ M)† | 165 |
| Boiled oxidase (0·1 ml.) | 167 |
| Vacuum-dried oxidase (4 mg.) | 180‡ |

* Precipitate of protein in manometer flask.
† Concentrations used by Schneider & Potter (1943).
‡ Corrected for $O_2$ uptake of dried preparation.

Pigeon-breast-muscle preparations, on the other hand, are not affected by proteins (Keilin & Hartree, 1947*b*).

### D. *The effect of cytochrome* c *compared with that of indifferent proteins*

The increased rate of oxidation of succinate by heart-muscle preparations obtained on adding proteins is of the same order as that given by cytochrome *c*. This raises the question whether cytochrome *c* may act as an indifferent protein as well as a specific oxido-reduction catalyst. The study of this question is most conveniently carried out with certain modified types of preparations which will be described below.

(1) *Experiments with frozen heart-muscle preparations.* The best results were given by a pig-heart preparation preserved 2–3 days at 5° after freezing 3 times in an ice-salt mixture. The rate of oxidation of succinate by this preparation was studied in presence of (*a*) horse-blood serum, (*b*) cytochrome *c* (0·43% Fe), (*c*) alkali denatured cytochrome *c*, (*d*) denatured globin and (*e*) denatured globin treated with alkali in the same way as the cytochrome *c*. The alkali treatment consists in 18 hr. contact with N-NaOH followed by neutralization with HCl and 4 hr. dialysis to remove excess of salt. We have previously shown (Keilin & Hartree, 1940) that such treatment renders cytochrome *c* catalytically inactive in an oxidizing system obtained from tissue extracts. The results of these experiments are summarized in Fig. 2 where the activating effects of various proteins are plotted against log protein concentration. The order of efficiency is: cytochrome *c* > denatured globin > serum proteins, but whereas alkali treatment reduces cytochrome *c* to the serum level, it does not affect denatured globin. These experiments distinguish clearly between the influence of added cytochrome *c* as an indifferent protein and its catalytic effect as an essential oxido-reduction catalyst. No explanation of the maxima in certain curves is forthcoming from our experiments.

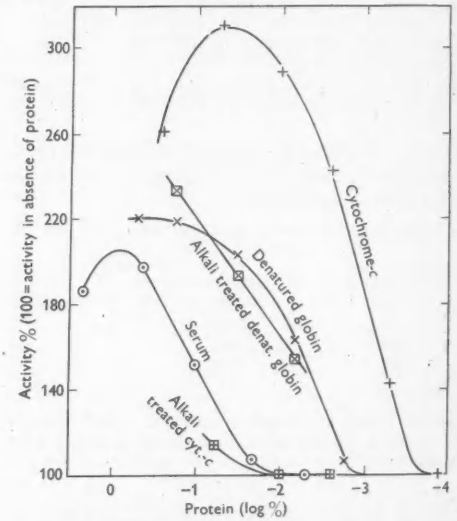



Fig. 2. Effect of cytochrome *c*, denatured globin and serum proteins on the oxidation of succinate by a frozen heart-muscle preparation (1·1 mg.). Ordinates: activity expressed as percentage of that in absence of the various proteins (36 $\mu$l. $O_2$/10 min.). Abscissae: log percentage protein concentration. Experiment carried out at 39° in 0·07 M-phosphate (pH 7·3).

(2) *Experiments with vacuum-dried heart-muscle preparations.* Horse-heart preparation (20 ml.) yielded 832 mg. powder on drying in the manner previously described. It was preserved in a desiccator for periods up to 4 weeks and resuspended in water (41·6 mg./ml.) as required. For manometric experiments 0·05 ml. of this suspension was used, while control runs were carried out with the corresponding amounts of undried material. The effect of phosphate concentration on this preparation is shown in Fig. 1. The results collected in Table 5 show that the activity of the preparation towards succinate is very much reduced by drying, when tested in 0·07 M-phosphate, and it is not reactivated by cytochrome *c*. Addition of denatured globin, however, increases the activity of the dried material to that obtained at the optimum phosphate concentration, and the addition of cytochrome *c* at this stage leads to a further increase in $O_2$ uptake. Cytochrome *c*, therefore, does not act here as an indifferent protein. An analogous oxidase

preparation from ox heart was less markedly affected by drying, probably owing to the presence of proteins which may function as denatured globin. In both cases, however, the effects produced by cytochrome *c* and by globin are additive. The results obtained with dried oxidase preparations in experiments of the type set out in Table 5 can vary considerably from one preparation to another and with the age of the preparation. Nevertheless, the distinction between the general effect of proteins and the specific effect of cytochrome *c* always remains clear.

Table 5. *Oxidation of succinate by heart-muscle preparations before and after drying. Effect of cytochrome* c *and denatured globin*

(Globin (0·3 ml. 1·4%); 0·07 M-phosphate buffer; temp., 39°; pH, 7·3.)

| | $O_2$ uptake ($\mu$l./15 min.) | | |
|---|---|---|---|
| | Horse heart | | Ox heart |
| | Fresh | Dried | (dried) |
| Oxid. + succ. | 78 | 2 | 62 |
| Oxid. + succ. + cyt. *c* | 98 | 9 | 102 |
| Oxid. + succ. + denat. globin | 193 | 80 | 145 |
| Oxid. + succ. + denat. globin + cyt. *c* | 220 | 174 | 252 |

(3) *Experiments with horse-kidney preparations.* The characteristic property of this type of preparation is that it oxidizes succinate or *p*-phenylenediamine very slowly unless cytochrome *c* is added, when the rate of $O_2$ uptake may increase at least 5-fold (Table 2). We have previously noted this marked effect of added cytochrome *c* upon kidney preparations and have suggested that it is due to a deficiency of cytochrome *c* (Keilin & Hartree, 1940). When the cytochromes in our kidney preparations are examined spectroscopically after reduction with succinate or $Na_2S_2O_4$, a feeble band (*a*) is seen together with a broad diffuse band ($b_1$), which occupies the same position as the two distinct bands (*b*) and (*c*) found in heart preparations. In the same paper we showed that it was possible to obtain heart preparations that were not deficient in cytochrome *c* and yet were virtually inactive in the aerobic oxidation of succinate unless soluble *c* was added. It is thus possible for *c* to be present in an unavailable form. In this connexion the results of Slater (1949) are of interest. He has found that if the acidification to pH 5·5 is avoided during the preparation of the kidney oxidase (see p. 206) and replaced by high-speed centrifuging as a means of concentrating the active material, a normal heart-type cytochrome spectrum can be observed on addition of succinate. However, the rate of oxidation of succinate by such material, which shows an optimal effect of [phosph], is also low in absence of added *c*; hence the *c* in the preparation must be largely inaccessible to other components of the catalytic system.

Experiments with our kidney preparation, under standard experimental conditions, are shown in Table 6. The distinction between the accelerating effects of cytochrome *c* and denatured globin is apparent from the fact that globin is ineffective in absence of added *c*.

Table 6. *The effect of various activating factors on the oxidation of succinate by a horse kidney preparation*

(Denatured globin (0·3 ml. 1·4%), $4 \times 10^{-4}$ M-$AlCl_3$, $10^{-3}$ M-methylene blue; 0·12 ml. kidney preparation, 0·07 M-phosphate buffer; temp., 39°; pH, 7·3.)

| Factor added | $O_2$ uptake ($\mu$l./30 min.) |
|---|---|
| None | 15 |
| Denatured globin | 22 |
| $AlCl_3$ | 21 |
| Meth. blue | 54 |
| Cyt. *c* | 111 |
| Cyt. *c* + globin | 172 |
| Cyt. *c* + $AlCl_3$ | 155 |

### E. *Effect of metals on the oxidation of succinate by different preparations*

Horecker, Stotz & Hogness (1939) have shown that low concentrations of salts of $Al^{+++}$, $Cr^{+++}$ and the rare earths markedly increase the rate of oxidation of succinate in presence of an ox-heart preparation. Swingle, Axelrod & Elvehjem (1942) found a similar activation of various tissue preparations by $Ca^{++}$ (see p. 210) while Schneider & Potter (1943) have studied the activation of an unwashed liver preparation by $Ca^{++}$ and $Al^{+++}$.

(1) *Effect of* $Ca^{++}$ *and* $Al^{+++}$. The influences of these metallic ions added separately on the aerobic oxidation of succinate by heart-muscle preparations are quite different from those recorded by Schneider & Potter who found that, in liver extracts, $Ca^{++}$ alone activated the system and that $Al^{+++}$ merely abolished an initial lag in $O_2$ uptake. In Fig. 3

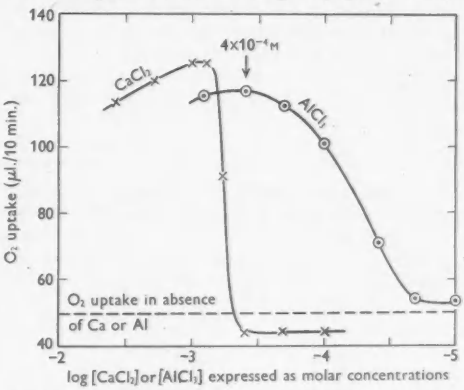



Fig. 3. Effect of different concentrations of $CaCl_2$ and $AlCl_3$ on the rate of $O_2$ uptake by 0·1 ml. heart-muscle preparation in presence of cytochrome *c* and succinate. 0·07 M-phosphate, pH 7·3; temp., 39°. Abscissae: log [$CaCl_2$] or [$AlCl_3$] expressed as molar concentrations.

are shown the effects of various concentrations of $CaCl_2$ and $AlCl_3$ on the aerobic activity of a horse-heart preparation under the standard conditions. It was of interest to find that the concentration at which the effect of $Ca^{++}$ disappears

is also the minimum concentration at which a gelatinous precipitate of $Ca_3(PO_4)_2$ is formed in the manometer flask. In the case of $Al^{+++}$ the minimum concentration could not be gauged as the precipitate forms very slowly at low $Al^{+++}$ concentration. At $4 \times 10^{-4}$ M, only $Al^{+++}$ is effective and the activation by the ions was found not to be additive. Anaerobic experiments with methylene blue gave no useful results owing to co-precipitation of the dye with the insoluble phosphate. The fact that the effects produced by $Ca^{++} + Al^{+++}$ and by globin are not additive suggests that their modes of action are similar. They both form precipitates in the manometric flasks, thus providing surfaces for possible adsorption, which corroborates the conclusion reached by Horecker *et al.* (1939), namely that the activation they obtained with $Cr^{+++}$ and other ions arose from the formation of precipitates of heavy metal phosphates. The dried oxidase preparations are also markedly reactivated by $Ca^{++}$ and $Al^{+++}$. Different results, however, were obtained when $CaCl_2 + AlCl_3$ were added to the oxidase preparation before drying. In such experiments, to 10 ml. heart-muscle preparation were added 0·43 ml. each of 0·6 M-$CaCl_2$ and 0·6 M-$AlCl_3$ in order that the final concentration of the two salts should be $4 \times 10^{-4}$ M in the subsequent manometer experiments. After vacuum drying and resuspension of the powder in 10 ml. water, the preparation was found to be completely inactive and further addition of $Ca^{++}$ and $Al^{+++}$ failed to reactivate it, whereas the control dried preparation behaved normally (Table 7).

Table 7. *Activity of succinic system in horse-heart preparations dried with or without the addition of* $CaCl_2$ *and* $AlCl_3$

($4 \times 10^{-4}$ M-$CaCl_2$ and $AlCl_3$; temp., 39°; pH, 7·3.)

| | $O_2$ uptake ($\mu$l./20 min.) |
|---|---|
| Oxid. dried with $Ca^{++}$ and $Al^{+++}$ + succ. + cyt. *c* | 0 |
| Oxid. dried with $Ca^{++}$ and $Al^{+++}$ + succ. + cyt. *c* + $CaCl_2 + AlCl_3$ | 0 |
| Oxid. dried alone + succ. + cyt. *c* | 17 |
| Oxid. dried alone + succ. + cyt. *c* + $CaCl_2 + AlCl_3$ | 109 |

Our results cannot be interpreted in the same manner as those of Swingle, Axelrod & Elvehjem (1942). By working with homogenates of unwashed tissues, these workers obtained evidence that the effect of $Ca^{++}$ arises from its activation of an enzyme hydrolyzing coenzyme I, the latter being an indirect inhibitor of succinic dehydrogenase (see p. 215). In fact we were able to show that a filtrate of boiled heart preparation was completely devoid of this coenzyme since it could not replace authentic coenzyme I in the reconstructed muscle lactic dehydrogenase system (Straub, 1940). Further arguments against such a possibility are: (1) that heart-muscle preparations in optimal [phosph] are not affected by $Ca^{++}$ since the latter merely raises the activity to the optimal level, and (2) no effect of $Ca^{++}$ can be observed when using enzyme material from other sources or when experiments are carried out in phosphate-free buffers (Table 8).

(2) *Effects of other metals.* The influence of other metals on the activity of a heart preparation in phosphate and in glyoxaline buffers and on a pigeon preparation in phosphate buffer is summarized in Table 8. In no case was there any activation of heart preparations in glyoxaline buffer or of pigeon preparations in phosphate buffer. The activations produced by $Ba^{++}$, $Fe^{+++}$, $Mn^{++}$ and $Ce^{+++}$ resemble those obtained with $Ca^{++}$ and $Al^{+++}$. Inhibition is always complete in the case of $Cu^{++}$, but variable in the case of $Co^{++}$. The formation of dense granular precipitates by $Ce^{+++}$, $Co^{++}$ and $Cu^{++}$ in glyoxaline accompanies complete inactivation. The lack of activation by metals of the pigeon preparation in phosphate buffer finds a parallel in the absence of any effect of proteins, whether native or denatured, under the same circumstances.

### F. *Evidence for the existence of a carrier linking succinic dehydrogenase with cytochrome*

Several workers claim to have demonstrated the existence of, or even to have isolated, a carrier linking succinic dehydrogenase with the cytochrome system. We shall now summarize the evidence

Table 8. *Effect of metals on the succinic system in horse heart and pigeon breast-muscle preparations*

(Heart preparation (0·05 ml.), 0·07 ml. pigeon preparation, 0·2 ml. cytochrome *c* in all cases. Metals ($2 \times 10^{-3}$ M) added as chlorides or sulphates; 0·07 M-buffer; temp., 39°; pH, 7·3.)

| | Preparation and buffer | | | | | |
|---|---|---|---|---|---|---|
| | Heart prep. in phosphate | | Heart prep. in glyoxaline | | Pigeon prep. in phosphate | |
| Metal added | Rate* | Ppt.† | Rate* | Ppt.† | Rate* | Ppt.† |
| None | (100) | — | (100) | — | (100) | — |
| $Ba^{++}$ | 171 | *I* | 96 | ‡ | 88 | *I* |
| $Ca^{++}$ | 208 | *I* | 108 | *O* | 86 | *I* |
| $Ce^{+++}$ | 181 | *I* | 0 | *D* | 85 | *I* |
| $Co^{++}$ | 68 | *S* | 0 | *D* | 43 | *S* |
| $Cu^{++}$ | 0 | *I* | 0 | *D* | 0 | *I* |
| $Fe^{+++}$ | 174 | *I* | 86 | § | 94 | *I* |
| $Li^{+}$ | 104 | *O* | 109 | *O* | 100 | *O* |
| $Mn^{++}$ | 175 | *I* | 109 | *O* | 110 | *I* |

* Relative initial rates of $O_2$ uptake.

† Key to letters and signs: *O*, no precipitate formed during experiment; *I*, immediate precipitate of gelatinous phosphate; *S*, slow formation of precipitate; *D*, slow formation of dense granular precipitate; ‡, slight crystalline precipitate of $BaSO_4$; §, precipitate of $Fe(OH)_3$.

brought forward in support of these claims and re-examine it in the light of our experiments.

(1) The first enzyme preparation with properties suggesting the existence of a carrier was obtained by Hopkins, Lutwak-Mann & Morgan (1939). They washed a mince of pig heart with sodium chloride and lithium chloride solutions and extracted it with bile salts. After precipitation of this extract with ethanol and extracting the precipitate with urea, a suspension was obtained which did not reduce cytochrome *c*, and which did not catalyze the oxidation of succinic acid by $O_2$ unless methylene blue was added. Unfortunately, this treatment denatures the components *a*, $a_3$ and *b* of cytochrome and inactivates the major part of the succinic dehydrogenase. In fact, the $Q_{O_2}$ of this preparation was about 12 whereas the $Q_{O_2}$ of an active heart preparation lies between 500 and 700. It has been shown (Keilin & Hartree, 1940) that 10 min. contact with 50% ethanol brings about the following changes in our heart muscle preparation: (1) the cytochrome oxidase activity, as measured by its ability to oxidize *p*-phenylenediamine, is abolished, (2) cytochromes *a*, $a_3$ and *b* are found, on spectroscopic examination, to be irreversibly modified, and (3) the succinic dehydrogenase activity, measured by the rate of $O_2$ uptake in presence of methylene blue, is reduced to about one third of its original value. The behaviour of the preparation of Hopkins *et al.* (1939) is thus best explained as being due to inactivation of the cytochrome system rather than to removal of an essential factor.

(2) By ultracentrifuging a heart-muscle preparation, Stern & Melnick (1939) obtained a preparation incapable of oxidizing succinic acid in presence of cytochrome *c*, although it contained an active succinic dehydrogenase, oxidizing the substrate by means of methylene blue, and an active cytochrome system as measured by the rate of oxidation of hydroquinone. However, when the supernatant fluid was added to the ultracentrifuged preparation, a complete system was obtained which catalyzed the aerobic oxidation of succinic acid. The supernatant fluid, according to them, contained 'a supplementary principle' or 'activator' which is a protein of molecular weight about 140,000. As we have mentioned previously (Keilin & Hartree, 1940), we were not in a position to repeat this experiment, but on centrifuging our heart preparation at a lower speed (13,000 r.p.m.) we obtained a sediment which was as active as the original material. As any factor which remained in solution at the higher speed must also have remained at our lower speed it is impossible to reconcile the two experiments on the basis of the separation of a component. The inactivation of Stern & Melnick's ultracentrifuged preparation cannot, therefore, be considered as being due to the separation of a carrier. It is probable that, under the influence of a high gravitational field, the preparation of Stern & Melnick underwent changes similar to those caused by drying or freezing. No such effect was observed in the lower gravitational field of our experiment.

We have already seen that marked activation of various types of enzyme preparation may be obtained with indifferent and denatured proteins. That Stern & Melnick's results may find an explanation along the same lines appears probable as a result of our experiments with dried heart and kidney preparations. We have shown (Table 5, second column of figures) that on drying a horse-heart preparation its catalytic activity towards succinic acid in presence of added cytochrome *c* is reduced to about 9% of that of the original material. In contrast to the inappreciable effect of cytochrome *c* on the dried preparation, the addition of denatured globin markedly raises the activity, and the further addition of cytochrome *c* at this stage more than doubles the activity so that it approaches 80% of that of the fresh preparation. The analogy with the results of Stern & Melnick becomes more striking when the activities of dried heart and kidney preparations are examined in presence of methylene blue, cytochrome and denatured globin. The results obtained with these heart and kidney preparations are shown in Table 9 and Fig. 4 respectively. It is seen that the activation by cytochrome *c* in such cases is very small and considerably less than that by methylene blue. In other words, a dried preparation catalyzes the oxidation of succinate by methylene blue (Fig. 4, iv) more efficiently than by cytochrome *c* (iii). In absence of added cytochrome *c* globin has no effect on this system (i and ii), but the simultaneous addition of globin and cytochrome *c* leads to a very marked activation (v). Whereas the addition of methylene blue to this last system does not increase its catalytic activity, an increase is obtained when either cytochrome *c*, globin, or both are absent (i*a*, ii*a*, iii*a*). Thus a non-specific and denatured protein appears to facilitate the reaction between dehydrogenase and the cytochrome system in the same way as the supplementary principle or activator of Stern & Melnick. The latter cannot, therefore, be considered, without further evidence, as a specific component of the catalytic system in tissues which oxidize succinic acid.

(3) Straub (1942) treated an extract of pig-heart muscle with sodium cholate and ammonium sulphate and resuspended the resulting precipitate in phosphate buffer. This 'cholate preparation' oxidized *p*-phenylenediamine, but not succinate unless methylene blue was added. It thus contained both the cytochrome system and succinic dehydrogenase, but apparently a certain factor linking the two was missing. A material which fulfilled this function

('SC factor') was obtained by heating a portion of the heart extract in alkaline solution. Straub considers the 'SC factor' to be an essential intermediate link in the cytochrome-succinic dehydrogenase system. This factor must be very different from the thermolabile protein 'activator' described by Stern & Melnick.

Table 9. *The effect of methylene blue and denatured globin on the succinic system in a dried horse-heart preparation*

(Denatured globin (0·3 ml., 1·4%), $10^{-3}$ M-methylene blue; dried oxidase, succinate, cytochrome *c*, 0·07 M-phosphate; temp., 39°; pH, 7·3.)

| Addition | $O_2$ uptake ($\mu$l./15 min.) |
|---|---|
| None | 11 |
| Meth. blue | 58 |
| Globin | 160 |
| Globin + meth. blue | 184 |

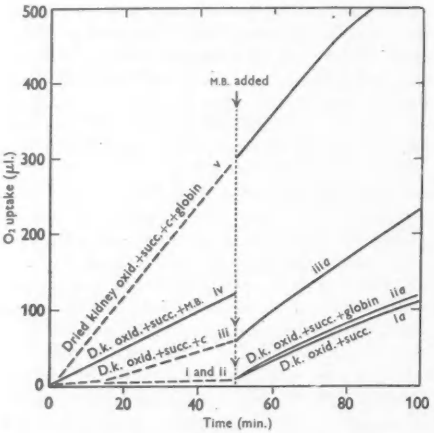



Fig. 4. Effect of cytochrome *c* (0·2 ml., 1%), denatured globin (0·3 ml., 1·4%) and methylene blue ($10^{-3}$ M) on the oxidation of succinate by a dried kidney oxidase preparation. Oxidase preparation equivalent to 0·1 ml. of material before drying. 0·07 M-phosphate buffer, pH, 7·3; temp., 39°.

We isolated the cholate preparation of Straub strictly according to his description, and we obtained his 'SC factor' by incubation of the heart-muscle preparation for 30 min. at pH 9 and 20° instead of at the higher temperature given in his paper which, in our hands, yielded a completely inactive material. Having confirmed Straub's results (Table 10, 1–4) we attempted to replace the 'SC factor' by substances which activated our enzyme preparations. Calcium phosphate was found to be much more efficient than the SC factor, whereas denatured globin or $Al^{+++}$ were completely ineffective (Table 10, 5–8). Such a striking difference between calcium phosphate and other activators was not observed with any of the enzyme preparations previously examined. The marked activating effect of $Ca^{++}$ in this case is apparently due to precipitation as calcium salt of the residual cholate. The low activity in Exp. 1 can, therefore, be reasonably ascribed to an inhibition of the catalytic system by sodium cholate present in this preparation. A notable increase in the activity of the 'SC factor' was found after 3 days' storage at 5°, although the dehydrogenase activity of the cholate preparation

Table 10. *The effect of* $Ca^{++}$ *and denatured globin on the succinic system prepared according to Straub* (1942)

(Cholate preparation (0·5 or 1·0 ml.), 1·0 ml. 'SC factor', 0·2 ml. 1% cytochrome *c*, 0·04 M-sodium succinate, 0·3 ml. $Ca_3(PO_4)_2$ gel (20 mg. dry wt.), 0·3 ml. 1·4% denatured globin, $10^{-3}$ M-methylene blue; temp., 39°; pH, 7·3.)

| | $O_2$ uptake ($\mu$l./10 min.) Tested same day using cholate prep. (1·0 ml.) | Tested 3 days later using cholate prep. (0·5 ml.) |
|---|---|---|
| 1. Chol. prep. + cyt. *c* + succ. | 7* | 2 |
| 2. Chol. prep. + cyt. *c* + succ. + 'SC factor' | 31† | 92 |
| 3. 'SC factor' + cyt. *c* + succ. | 0 | 2 |
| 4. Chol. prep. + meth. blue + succ. | 342 | 145 |
| 5. As 1 + $Ca_3(PO_4)_2$ | 232† | — |
| 6. As 1 + denatured globin | 2 | — |
| 7. As 2 + $Ca_3(PO_4)_2$ | 231 | — |
| 8. As 2 + denatured globin | 33 | — |

* This system is not activated by $10^{-3}$ M-$AlCl_3$ nor by increasing [phosph].

† $O_2$ uptake slows down after first 10 min.

decreased during the same interval. This change coincides with an increased aggregation and precipitation of the proteins in the factor which may then act as a more efficient adsorbent of cholate. It is, therefore, probable that the 'SC factor' does not represent a specific link in the catalytic chain of the system oxidizing succinate, but that it merely serves to remove cholate from solution. On the other hand, since we obtained the 'SC factor' under conditions that were slightly different from those specified by Straub, further study will be required before the mechanism of its action can be definitely established (see also Slater, 1949).

(4) By fractionating an aqueous extract of minced ox or rabbit liver with ammonium sulphate Stoppani (1947) isolated a preparation which, although it contained both succinic dehydrogenase and the complete cytochrome system, was yet incapable of catalyzing the aerobic oxidation of

succinate even in presence of added cytochrome *c*. This material was precipitated from the liver extract within the range 0–0·2 saturation with ammonium sulphate. However, when the fraction which precipitated between 0·33 and 0·6 saturation was purified through calcium phosphate it was found to have a strong activating effect on the first fraction in the aerobic oxidation of succinate. One of the peculiarities of the first fraction as a succinic dehydrogenase was that it could utilize ferricyanide as H acceptor but not methylene blue. The activator was easily destroyed by heat and by acids and would appear to have been a protein. It could not be replaced by $10^{-5}$ M-$Ca^{++}$ or $Al^{+++}$ or by different coenzymes.

In our attempts to repeat Stoppani's experiments, following his methods as closely as possible, we were unable to obtain fractions which showed a differentiation in the reactions with ferricyanide and methylene blue: in fact all fractions that we obtained between 0 and 0·2 saturation readily reduced methylene blue anaerobically in presence of succinate. On one occasion we were able to obtain from rabbit liver two fractions precipitated within the specified ammonium sulphate concentrations which resembled Stopanni's materials. Details of this experiment are set out in Table 11, where it is seen that the factor may be replaced by $Ca^{++}$ or horse serum but not by $Al^{+++}$ or denatured globin. We have used much higher concentrations of $Ca^{++}$ and $Al^{+++}$ than did Stoppani, which may well explain the discrepancy (cf. Fig. 3).

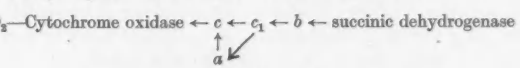
Table 11. *Effect of* Ca, Al *and proteins on the succinic system prepared according to Stoppani* (1947)

(Enzyme preparation, rabbit liver (0·5 ml.), 1 ml. 'factor' (rabbit liver). 0·2 ml. 1% cytochrome *c* (in all experiments), $10^{-3}$ M-methylene blue, 0·005 M-$AlCl_3$, 0·005 M-$CaCl_2$, 0·14% denatured globin, 0·3 ml. horse serum. 0·04 M-sodium succinate, 0·07 M-phosphate buffer; temp., 39°; pH, 7·3.)

| | $O_2$ uptake ($\mu$l./hr.) |
|---|---|
| 1. Enzyme + succinate | 3 |
| 2. 'Factor' + succinate | 3 |
| 3. Enzyme + 'factor' + succinate | 124 |
| 4. As 1 + $Ca^{++}$ | 107 |
| 5. As 1 + $Al^{+++}$ | 8 |
| 6. As 1 + serum | 87 |
| 7. As 1 + denatured globin | 21 |
| 8. As 1 + meth. blue | 76 |

Our experiments suggest that the activating material isolated by Stoppani contains no essential link in the catalytic system oxidizing succinate, but comes within the category of indifferent proteins which can have a marked effect upon our heart-muscle and kidney preparations.

(5) Yakushiji & Okunuki (1940) have described a haem-protein of haemochromogen type under the name cytochrome $c_1$ and consider it to be a catalytic intermediate in the cytochrome system. They repeatedly washed an isoelectric precipitate of a heart preparation with acetate buffer, pH 4·6, before suspending it in phosphate at pH 7·5 and found that, on reduction with succinate + HCN, it showed an absorption band at 552 m$\mu$. This pigment, which can be extracted from heart preparations in a soluble form, will reduce cytochromes *a* and *c*. These authors suggest that it acts as an oxido-reduction carrier between cytochrome *b* and cytochromes *a* and *c*:




(arrows indicate direction of electron transfer)

However, since $c_1$ can only be detected or isolated after treatments which have an adverse effect upon the activity of the complete succinic system (e.g. sodium cholate or ether) the possibility that it is an artifact derived from other haem-proteins (see p. 214) must be seriously considered. On the other hand, the function postulated for this substance is very similar to that put forward by Slater (see below) for a labile haem-protein detected by him in heart-muscle preparations, and it is possible that the two 'factors' may be closely related.

(6) While studying the effect of BAL (2:3-dimercaptopropanol) on the succinic dehydrogenase-cytochrome system, Slater (1948, 1949) has obtained a heart-muscle preparation exhibiting interesting properties. Although this preparation failed to catalyze the aerobic oxidation of succinic acid, it showed an almost unimpaired activity both of succinic dehydrogenase, as shown by the rate of oxidation of succinate in presence of methylene blue, and of the cytochrome system estimated by the oxidation in presence of cytochrome *c* of ascorbic acid, hydroquinone or *p*-phenylenediamine. On addition of succinate to such a preparation, cytochrome *b* becomes permanently reduced, whereas the other cytochrome components remain in the oxidized state, an effect resembling that observed on addition of urethane to a normal preparation (Keilin & Hartree, 1939, 1940). On addition of $Na_2S_2O_4$, the reduced components of cytochrome (*a*, $a_3$, *b*, *c*) appear normal, although the total protohaematin content of the preparation, determined by addition of pyridine plus $Na_2S_2O_4$ and measurement of the intensity of the haemochromogen band at 548–560 m$\mu$, is 20% below that of the heart preparation before treatment with

BAL. Slater's interpretation of these results is that the heart preparation contains a BAL-labile factor which is an intermediate in the electron transfer from cytochrome *b* to cytochrome *c* and which is probably a haematin derivative. The system may be represented as follows:

$O_2$—cytochrome oxidase ← *a* ← *c* ← factor ← *b* ← succinic dehydrogenase.

The preparation obtained by Slater shows some resemblance to two preparations that we obtained by incubating heart-muscle oxidase either at pH 5·0 or at a higher pH in presence of pancreatin (Keilin & Hartree, 1940). In all cases there appears to be a lack of interaction between the succinic dehydrogenase on the one hand and the cytochrome system on the other. We suggested that the impairment of the aerobic succinoxidase activity could be explained, either by loss of a factor intermediate between dehydrogenase and cytochrome *c* or by an irreversible change in the colloidal structure of the preparation, with a consequent decrease in the mutual accessibility of the catalysts in the system.

Although we cannot exclude the possibility of the existence of several further intermediate links between the known components of the succinic dehydrogenase-cytochrome oxidase system, we have seen that much of the evidence is inconclusive, and so far only that brought forward by Slater appears to be well founded upon experimental evidence.

## DISCUSSION

The tissue preparations used for the study of the system catalyzing the aerobic oxidation of succinic to fumaric acid consist of colloidal suspensions of protein aggregates. In addition to the complete succinic dehydrogenase-cytochrome system these preparations also contain fumarase together with small concentrations of catalase and certain dehydrogenases (e.g. malic and lactic); they are, however, free from haemoglobin and coenzymes I and II. Being completely devoid of substrate, they have no $O_2$ uptake, show cytochromes in a completely oxidized state and do not reduce methylene blue anaerobically. After addition of sodium succinate the cytochromes rapidly become reduced and on aerating the mixture they re-oxidize only to become again reduced when aeration ceases. The preparation will now take up $O_2$ rapidly and will reduce methylene blue anaerobically.

Spectroscopically the preparation shows the cytochrome components *a*, $a_3$, *b* and *c* (Keilin & Hartree, 1939). It also contains other forms of haematin which were previously described as free haematin (Keilin, 1925), but which we now consider to be present as one or more haematin-protein derivatives (Keilin & Hartree, 1947*b*). That such compounds are not visible spectroscopically may be due to their diffuse absorption bands. However, on treating these preparations with $Na_2S_2O_4$ and pyridine, the extra haematin becomes spectroscopically visible as pyridine haemochromogen, the α band of which is clearly seen obliterating the space between the α bands of cytochromes *b* and *c*.

Since the activity of the complete succinic system can be studied both spectroscopically and manometrically, we are able to localize the actions of a number of inhibitory or activating factors and to determine the mechanism of their action. Further analysis of this system can be made by independent study of the activities of its two portions. Thus the activity of succinic dehydrogenase can be determined either anaerobically by the rate of reduction of methylene blue, or aerobically, by the rate of $O_2$ uptake in presence of methylene blue and of cyanide which inhibits the cytochrome oxidase. The activity of the cytochrome system can readily be followed manometrically by the rate of oxidation of substances which reduce cytochrome *c* directly, i.e. without the need of specific enzymes. Such substances are ascorbic acid, cysteine, phenols and aromatic amines.

Whereas some factors act on one or other of the known components of the system others affect either some, as yet undetermined, intermediate components, or the colloidal structure of the preparation which assures the mutual accessibility of the components. In any attempt to analyze the complex systems represented by our colloidal preparations it is important to determine not only the point at which the factor reacts but also its mode of action. Even if we confine our attention to inhibitors we find that in some cases the mode of action is easily demonstrated, while in others it requires a more thorough investigation. Thus the powerful, instantaneous and perfectly reversible inhibition of the system by HCN, $NaN_3$ or $H_2S$ is due to their combination with the trivalent Fe of cytochrome oxidase ($a_3$), while the effect of CO depends upon its ability to combine with oxidase Fe in the divalent state. All these reactions can be followed spectroscopically (Keilin & Hartree, 1939). On the other hand, malonate (Quastel & Wooldridge, 1928), oxaloacetate (Das, 1937) and pyrophosphate (Dixon & Elliott, 1929; Leloir & Dixon, 1937) act as competitive inhibitors of succinic dehydrogenase. The relative affinity of this enzyme ($E$) for substrate ($s$) and inhibitor ($i$) determined experimentally and expressed as a partition constant $K = [Ei][s]/[Es][i]$ is about 13 for malonate, 23 for pyrophosphate and 850 for oxaloacetate. Spectroscopic study of enzyme preparations shows that these inhibitors prevent the reduction of all components of cytochrome by succinate.

On the other hand the mechanisms of the inhibition of this system by coenzyme I and by sodium diethyldithiocarbamate (DDC) are less direct and require a more careful consideration. The inhibition of aerobic oxidation of succinate by coenzyme I was first recorded by Potter (1939). We confirmed this effect and found a marked inhibition at coenzyme concentrations as low as $0{\cdot}5 \times 10^{-5}$–$2{\cdot}5 \times 10^{-5}$ M. At first sight this inhibition could be ascribed to a direct reaction of coenzyme with some reactive groups of the dehydrogenase. However, a study of this reaction under varying conditions revealed the following mechanism (Keilin & Hartree, 1940). Since heart-muscle preparations contain fumarase and malic dehydrogenase, part of the fumarate arising from the oxidation of succinate is transformed to malate which, in presence of coenzyme, is oxidized to oxaloacetate. The latter, being neither reduced nor decarboxylated at an appreciable rate, acts as a competitive inhibitor of succinic dehydrogenase. In fact, if the concentration of oxaloacetate reaches only 0·2 % of that of the succinate it would already inhibit the $O_2$ uptake by > 50 % (see p. 214).

The study of the inhibition by DDC is of special interest. This substance is chiefly known as a reagent for the colorimetric estimation of copper and will act as an inhibitor of reactions catalyzed both by $Cu^{++}$ ions and by copper-protein enzymes such as polyphenol oxidase. The inhibition by this substance of the aerobic oxidation of succinate at first suggested the possibility that a copper derivative participated in the reaction. However, a further study of this reaction clearly showed that the inhibition was not due to DDC itself but to its oxidation product tetraethyldithiocarbamyl disulphide (TDD) $(C_2H_5)_2NCS.SS.SCN(C_2H_5)_2$ derived from DDC through an oxidation catalyzed by the cytochrome system, since DDC reduces cytochrome *c* (Keilin & Hartree, 1940). In fact it was found that while DDC inhibited succinate oxidation only after it had become oxidized, TDD, in concentrations as low as $5 \times 10^{-6}$ and $2 \times 10^{-5}$ M, inhibited $O_2$ uptake immediately to the extent of 70 and 100 % respectively.

Since the demonstration by Hopkins & Morgan (1938) that the activity of succinic dehydrogenase depends upon the integrity of certain —SH groups in the enzyme, the mechanism of action of a number of inhibitors has been explained by their ability to combine with —SH groups or to oxidize them (see Potter & DuBois, 1943; Slater, 1949).

The activity and behaviour of the succinic system show considerable variations both with the type of tissue used and with the species of animal from which it was obtained. The effects of different factors, such as phosphate concentration, native and denatured proteins and salts of a number of metals, were tested both on fresh enzyme preparations and on vacuum-dried and frozen samples.

The study of the influence of phosphate concentration on the activity of a heart preparation has demonstrated a relationship between activity and the degree of colloidal dispersion. We have not attempted to express the degree of dispersion in any quantitative form, but have regarded the time taken for the proteins in a preparation to flocculate spontaneously and settle out under gravity as a rough measure of the degree of dispersion. Thus the diluted heart-muscle extract described on p. 206, which remains homogeneous for several hours, is regarded as completely dispersed. Maximum dispersion and enzyme activity are obtained in 0·15 M-phosphate (pH 7·3) with an increasing flocculation and falling off in activity as the concentration deviates from this figure. When the total salt concentration remains constant and [phosph] is varied, an activating effect of phosphate is detectable and the occurrence of an optimum concentration is apparently due to two opposing influences: peptization and salting out. In our kidney preparation the former influence is absent; hence the activity increases continuously with diminishing [phosph]. A point of certain interest is that fully active heart preparations can be obtained in absence of phosphate, glyoxaline replacing phosphate during the extraction from the muscle and in the manometers.

We have shown that, contrary to the views of Biró & Szent-Györgyi jun. (1946), ATP does not activate, but slightly inhibits the system oxidizing succinic acid. If any link exists between this system and ATP it must be indirect and may depend upon catalytic systems not present in our preparations. Moreover, even if the synthesis of ATP is linked with the oxidation of succinate it does not logically follow that ATP should have any effect upon the activity of the succinic dehydrogenase-cytochrome system.

The importance of colloidal structure for the activity of the complete system is clearly demonstrated by the fact that when [phosph] is reduced to a suboptimal value, e.g. 0·07 M, the addition of denatured globin or of $Ca^{++}$ or $Al^{+++}$ brings the activity of the system to the level shown at the optimum [phosph] (Table 3). These additions give rise to bulky precipitates on which the enzyme preparation becomes adsorbed although similar, but less marked, effects are observed with proteins that remain in solution (Fig. 2 and Table 4). The effect of denatured globin is even more striking in the cases of dried heart and dried kidney preparations (Table 5 and Fig. 4). Where the metals do not form bulky precipitates (e.g. in glyoxaline buffer) no activation is obtained while a lack of activation in phosphate buffer by either proteins or metals is observed if the enzyme preparation already consists

of a bulky flocculent precipitate as in the case of pigeon-breast muscle (see Keilin & Hartree, 1947*b*, table 2; also Table 8 of this paper).

We have seen that the activation of the system by proteins generally and by cytochrome *c* may be of the same order of magnitude. This naturally raises the question of the exact function of added *c* in our experiments. That it acts as an oxido-reduction catalyst and not as an indifferent protein is clearly demonstrated by the following observations:

(1) When cytochrome *c* is denatured by treatment with alkali it becomes autoxidizable and, at the same time, catalytically inactive (Keilin & Hartree, 1940). Such material fails to accelerate the $O_2$ uptake of a heart preparation oxidizing succinate at a concentration equal to the optimal concentration of native *c* (Fig. 2).

(2) Unlike indifferent proteins or metallic ions, cytochrome *c* markedly increases the rate of oxidation of succinate by fresh kidney preparations (Table 6).

(3) The above proteins and ions may activate some preparations to an equal or even greater degree than does cytochrome *c*. However, the effects upon $O_2$ uptake induced by the indifferent activators and by *c* are additive or even synergic (Table 5).

(4) Dried enzyme preparations from horse heart or kidney show a very low catalytic activity towards succinic acid. The reactivation of a dried horse-heart preparation by denatured globin is usually greater than that produced by cytochrome *c*. However, when both substances are added simultaneously, the resulting activity is markedly greater than the sum of the activities in presence of each of these substances acting separately. The behaviour of such preparations can, however, vary quite widely (see p. 209). A dried kidney preparation (Fig. 4), which fails to oxidize succinate, cannot be reactivated by proteins. Some reactivation is obtained with *c*, but methylene blue is more effective. On the other hand, denatured globin and cytochrome *c* added together will produce a very striking increase in the activity of the dried preparation, and bring it almost to the level of activity of the fresh material with the same additions.

It was previously shown (Keilin & Hartree, 1940, 1945) that the catalytic activity of cytochrome *c* within intact cells and tissues or their colloidal extracts is much greater than that of *c* added in soluble form. The experiments described in this paper lend considerable support to our view that the high activity of the succinic system in such extracts is due largely to the retention within the colloidal particles of the favourable spatial organization of catalysts characteristic of the cell. On such a hypothesis the much lower catalytic activity of added *c* is understandable. Moreover, the loss in activity of a tissue preparation treated in different ways, even when it can be made good by the addition of *c*, is less likely to be due to loss of endogenous *c* than to changes in colloidal structure which diminish the accessibility of endogenous *c* to other components of the system. The changes resulting from the various treatments can be reversed, and the system as a whole reactivated by addition of factors having an influence on the colloidal structure, such as proteins, ions yielding insoluble phosphates, and changes in [phosph]. An explanation of this reactivation can only be proposed in very general terms. Thus the factors may be responsible either for reorientation of the components, especially of endogenous cytochrome *c*, within the colloidal particles or for providing new reactive surfaces to which catalysts, in particular exogenous *c*, may become anchored. On these grounds the wide variations in response to these factors from one preparation to another are understandable.

The more or less complete loss of accessibility of cytochrome *c* may show different degrees of reversibility. In the extreme case of the dried kidney preparation the removal of *c* from the sphere of influence of other components is almost irreversible since it is impossible to reactivate the succinic system by addition of $Ca^{++}$ or proteins, and, furthermore, added *c* cannot be utilized catalytically unless such factors are also added. This preparation is of special interest since it resembles those obtained by several workers in their attempts to demonstrate the existence of new intermediary links between succinic dehydrogenase and cytochrome *c*. Such preparations share the property of being more strongly activated by methylene blue than by cytochrome *c*, and of being very highly activated when the latter is supplemented by another factor isolated from the same tissue. Although we have seen that it is not necessary to postulate the absence from the dried kidney preparation of a specific link in the succinate oxidizing system, one cannot rule out the possibility that one or more additional links between known components of the succinic dehydrogenase-cytochrome system do, in fact, exist. However, we have shown that the evidence brought forward is inadequate in all cases with one possible exception. The only reasonable evidence is that of Slater (1948), who postulates a specific link, probably of haematin nature, acting as an oxido-reduction carrier between cytochromes *b* and *c*. So far this conclusion is based solely upon an irreversible destruction of the factor and not upon its separation from an active preparation.

The activating effects that we have obtained may be summarized as follows. Marked changes in catalytic activity are associated with the introduction of bulky precipitates. If such precipitates are formed by addition of metallic salts giving gelatinous phosphates, or of proteins, an increase in activity may

be observed. If, on the other hand, precipitates are formed from the enzyme material itself there is generally a loss of activity (e.g. freezing or drying). Lost activity can frequently be restored by formation of precipitates of the activating type.

Treatments which reduce the activity by modifying the colloidal structure may (1) make endogenous cytochrome *c* unavailable or (2) make both endogenous and added *c* unavailable to the rest of the system. It is because the latter effects may often be reversed by indifferent proteins that claims for the existence of new 'links' in the succinic system have been made. Similarly, activation by $Ca^{++}$ of our preparation is not due to an accelerated removal of coenzyme I.

Previous evidence for new 'links' and for the function of $Ca^{++}$ was in each case based upon one type of preparation which was studied under a fixed set of conditions. Our results, however, show that a slight modification of these conditions may, through changes in colloidal structure, lead to fundamentally different results.

We may fittingly conclude by quoting from an earlier paper on the same subject (Keilin & Hartree, 1940). 'The succinic dehydrogenase-cytochrome system even in the cell-free colloidal preparations behaves as a true respiratory system of the cell, showing a high catalytic activity and being affected by all inhibitors in the same way and to the same degree as the normal respiration of intact cells. The efficiency of the system depends, however, not only on the integrity of its components, but also on that of the colloidal structure which supports them and assures their mutual accessibility.'

## SUMMARY

1. A study of the succinic dehydrogenase-cytochrome system in various types of tissue extract has been made, with special reference to the effect of colloidal structure on enzyme activity.

2. Heart-muscle preparations show optimum activity in 0·15 M-phosphate; at lower phosphate concentrations addition of proteins or certain metallic ions raises the activity to the optimum level. In this respect insoluble denatured proteins are the most effective. The activity of these preparations falls considerably after freezing, but it can be restored by addition of cytochrome *c*. The pronounced loss of activity that accompanies vacuum drying can be fully reversed only by adding both cytochrome *c* and either proteins or certain metal salts.

3. Kidney preparations are feebly active unless cytochrome *c* is added. They are virtually inactive after vacuum drying, but can be restored to full activity by addition of both cytochrome *c* and protein, although each added separately has very little effect. The activity of these preparations falls as the buffer concentration is increased; there is no optimum concentration.

4. The activation by metals is obtained in phosphate buffer, where gelatinous precipitates are formed, but not in glyoxaline buffer.

5. It is considered that the catalysts in the colloidal particles, as in the intact cells, are more or less rigidly held together within a framework which ensures their mutual accessibility and a consequent high catalytic activity. Any change in colloidal structure will therefore modify the overall activity without necessarily removing or destroying any individual catalyst.

6. The inhibitory effects of freezing, drying, extreme [phosph], are intimately bound up with the formation from the enzyme material of flocculent precipitates and a consequent decrease in inter-availability. Activation, however, arises when flocculent precipitates of foreign materials (proteins, gelatinous phosphates) are formed in the medium. Such material provides new surfaces upon which reorientation can take place. Since the pigeon preparations already consist of flocculent material they do not undergo activation.

7. It has been clearly demonstrated that added cytochrome *c* acts in these experiments solely as an oxido-reduction catalyst and not as a protein affecting the colloidal structure.

8. The various claims to have demonstrated the existence of new 'links' in the succinic system (comprising dehydrogenase, cytochromes *b*, *c*, *a* and $a_3$) have been examined in detail. All are shown to be based upon insufficient evidence except that of Slater (1948), who has produced reasoned arguments in favour of an oxido-reduction catalyst linking *b* and *c*.

9. Since our preparations are devoid of coenzyme I the view that its activation by $Ca^{++}$ arises from an accelerated decomposition of this coenzyme, as is the case with fresh homogenates, is untenable. Furthermore, $Ca^{++}$ is ineffective with certain types of tissue preparation.

10. Two types of factor (activating or inhibiting) can be distinguished. They may function either by reacting specifically with a catalyst in the system or by affecting the colloidal structure of the enzyme material. Activation of the latter type has often been misinterpreted as evidence for additional carriers or 'links' in the succinic dehydrogenase-cytochrome system.

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# The Component Acids of some Seal Blubber and Liver Fats


By T. P. HILDITCH and S. P. PATHAK
*Department of Industrial Chemistry, University of Liverpool*


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The fatty oils of seals have received little detailed investigation, but their general analytical characteristics as recorded in the literature (cf. Halden & Grün, 1929) reveal considerable variations in the blubber oils, iodine values from 122 to 163 having been reported. This unusually wide range is reflected in the two specimens of seal blubber oil which have so far been examined in greater detail, namely, a commercial sample (probably of Newfoundland origin) with an iodine value of 135·8 (Burke & Jasperson, 1944) and the blubber fat of a grey Atlantic seal (*Halichoerus grypus*) caught off the coast of Pembroke, with an iodine value of 162·2 (Hilditch & Pathak, 1947). The chief differences observed in the detailed analyses of these two seal oils consisted in variation in the proportions of unsaturated $C_{16}$ acids, unsaturated $C_{18}$ acids, and unsaturated $C_{22}$ acids.

When Prof. A. N. Worden of the University College of Wales, Aberystwyth (who had very kindly put material from the grey Atlantic seal at our disposal earlier), informed us that similar specimens from two common seals (*Phoca vitulina* L.) were to be available, it was felt desirable to undertake similar examination of their blubber and liver lipids in order to add to the somewhat meagre knowledge of the fatty acids present in the fats of members of this family. Prof. Worden was accordingly good enough to secure for us samples of blubber and of liver from two common seals taken off the Norfolk coast in July 1947, the lipids from which were extracted and examined in our laboratory.

## METHODS

The tissues (blubber or liver) were cut into small pieces and extracted exhaustively with hot acetone until no further lipid was removed. The material left after removal of the acetone was taken up in light petroleum (b.p. 40–60°), the solution separated from the water which had also been entrained by the acetone, and the lipids recovered. The liver lipids contained appreciable quantities of phosphatides as well as glycerides, and these were separated by keeping their acetone solutions at 0° for several days, when the phosphatides were precipitated. The component fatty acids of the blubber oils and (so far as the quantities available permitted) of the liver glycerides and phosphatides were determined by the methods used in our earlier work (Hilditch & Pathak, 1947) on the blubber and liver oils of the grey Atlantic seal. These are briefly as follows:

(*a*) The mixed fatty acids from the oil were first partly resolved by crystallization from acetone and from ether at

low temperature. Crystallization from 10% solution in acetone at −60° left in solution nearly all of the polyethenoid acids of the $C_{20}$ and $C_{22}$ series, with some oleic and much of the hexadecenoic acid; in one instance further quantities of the more unsaturated acids were obtained by subsequent recrystallization from acetone at −60° of the insoluble and soluble portions obtained in the first crystallization. In all instances the acids finally left insoluble in acetone at −60° were crystallized from 10% solutions in ether at −40°, when material of very low iodine value was deposited, leaving in solution a concentrate of mainly monoethenoid acids, accompanied by small amounts of saturated acids and polyethenoid acids of the $C_{20}$ series.

(*b*) Each group of acids thus obtained was separately converted into methyl esters, which were fractionally distilled in a vacuum through an electrically heated and packed column. From the iodine values and equivalents of the resulting ester fractions the compositions of the latter were calculated by methods which have been described elsewhere (Hilditch, 1947). The mean unsaturation, expressed by the fractional number of hydrogen atoms short of saturated, e.g. −2·0 (monoethenoid), was determined by interpolation or extrapolation from the respective ester fractions in each group from which the mean equivalent of each of the homologous ester groups ($C_{16}$, $C_{18}$, $C_{20}$ and $C_{22}$) follows. In this manner the fatty acid composition of each separated group of fatty acids, and thence that of the original seal blubber or liver lipid, was finally obtained.

## RESULTS

The animals from which the lipids were obtained were: I, a female about 2 or 3 weeks old, 915 mm. long (snout to tip of tail), girth at axilla 510 mm.; II, a yearling male, 1020 mm. long, girth at axilla 735 mm. The specimens will be referred to as I and II throughout this paper.

### *Blubber oils*

The blubber fats were almost wholly glyceridic and contained no phospholipids separable by deposition from acetone at 0°. They were pale yellow oils with the following analytical characteristics:

| | Oil of seal I | Oil of seal II |
|---|---|---|
| Iodine value | 140·0 | 145·4 |
| Saponification equivalent | 289·0 | 292·3 |
| Free fatty acid (as oleic) (%) | 0·2 | 0·1 |
| Unsaponifiable matter (%) | 0·1 | 0·3 |

*Component acids of blubber oil from seal I.* The mixed fatty acids (186 g.) were crystallized first from acetone at −60°, the deposited acids then being further crystallized from ether at −40°:

| Group | Description | Weight (g.) | Weight (%) | Iodine value |
|---|---|---|---|---|
| *A* | Insoluble in ether at −40° | 25·7 | 13·8 | 4·9 |
| *B* | Soluble in ether at −40° | 75·4 | 40·6 | 99·6 |
| *C* | Soluble in acetone at −60° | 84·9 | 45·6 | 218·1 |

The ester-fractionation data for the methyl esters of each of these three groups of acids are summarized in Table 1, and the values allotted to

Table 1. *Fractionation of methyl esters of seal I blubber oil acids A, B and C*

| Fraction | Weight (g.) | Saponification equivalent | Iodine value |
|---|---|---|---|
| | Methyl esters of acids *A* | | |
| *A*1 | 2·89 | 247·6 | 0·4 |
| *A*2 | 3·73 | 269·0 | 1·9 |
| *A*3 | 3·50 | 269·8 | 2·1 |
| *A*4 | 3·56 | 271·5 | 2·8 |
| *A*5 | 3·77 | 271·9 | 3·0 |
| *A*6 | 3·61 | 282·6 | 3·6 |
| *A*7 | 3·54 | 313·5* | 23·9 |
| | 24·60 | | |
| | Methyl esters of acids *B* | | |
| *B*1 | 2·82 | 254·2 | 52·2 |
| *B*2 | 4·20 | 266·8 | 85·9 |
| *B*3 | 4·66 | 266·9 | 88·1 |
| *B*4 | 3·87 | 273·0 | 88·4 |
| *B*5 | 5·11 | 280·7 | 88·6 |
| *B*6 | 5·30 | 294·3 | 88·6 |
| *B*7 | 5·54 | 295·1 | 89·0 |
| *B*8 | 4·99 | 296·2 | 89·2 |
| *B*9 | 3·98 | 296·8 | 92·3 |
| *B*10 | 3·73 | 305·7 | 92·9 |
| *B*11 | 3·25 | 313·1 | 117·4 |
| *B*12 | 3·05 | 321·3* | 152·8 |
| | 50·50 | | |
| | Methyl esters of acids *C* | | |
| *C*1 | 4·12 | 247·1 | 92·1 |
| *C*2 | 3·28 | 268·4 | 99·7 |
| *C*3 | 3·90 | 270·1 | 103·2 |
| *C*4 | 4·68 | 275·7 | 109·7 |
| *C*5 | 4·08 | 281·7 | 120·5 |
| *C*6 | 3·86 | 283·3 | 123·0 |
| *C*7 | 3·92 | 285·2 | 125·0 |
| *C*8 | 3·52 | 287·1 | 151·0 |
| *C*9 | 4·09 | 311·1 | 236·7 |
| *C*10 | 6·16 | 330·9 | 330·7 |
| *C*11 | 4·58 | 335·1 | 380·6 |
| *C*12 | 4·50 | 338·6 | 373·5 |
| *C*13 | 3·10 | 346·8* | 334·7 |
| | 53·79 | | |

* Equivalents of esters (freed from unsaponifiable matter): *A*7, 303·7; *B*12, 314·6; *C*13, 342·7.

Table 2. *Equivalents, iodine values and mean unsaturation employed in calculating the composition of unsaturated methyl ester fractions from groups B and C*

| Methyl esters of acids | Saponification equivalent | Iodine value | Mean unsaturation |
|---|---|---|---|
| | Group *B* | | |
| $C_{14}$ | 240·0 | 105·8 | − 2·0 |
| $C_{16}$ | 268·0 | 94·8 | − 2·0 |
| $C_{18}$ | 295·9 | 90·1 | − 2·1 |
| $C_{20}$ | 321·6 | 173·8 | − 4·4 |
| $C_{22}$ | — | — | — |
| | Group *C* | | |
| $C_{14}$ | 240·0 | 105·8 | − 2·0 |
| $C_{16}$ | 267·9 | 99·5 | − 2·1 |
| $C_{18}$ | 294·0 | 172·8 | − 4·0 |
| $C_{20}$ | 319·0 | 270·3 | − 7·0 |
| $C_{22}$ | 343·0 | 407·3 | −11·0 |

the various homologous groups of unsaturated $C_{14}$, $C_{16}$, $C_{18}$, $C_{20}$ and $C_{22}$ acids in each series are shown in Table 2.

The percentage composition (by weight) of each group of acids was then calculated, and therefrom the composition of the total fatty acids of the blubber oil (Table 3).

*Component acids of blubber oil from seal II.* In this case the first crystallization of the mixed fatty acids (235·5 g.) of the oil from acetone at $-60°$ caused 90·9 g. (iodine value 70·1) to be deposited, and left in solution 144·6 g. (iodine value 202·8). The latter acids, on repetition of this process (acetone at $-60°$), deposited 40·7 g. (*D*, iodine value 84·1) and left in solution 103·9 g. (*E*, iodine value 250·2). The acids deposited in the original crystallization were also recrystallized from acetone at $-60°$, when there were left in solution 20·1 g. (*C*, iodine value 145·3), and 70·8 g. (iodine value 47·0) were deposited and then crystallized from ether at $-40°$. This led to the separation of 32·5 g. (*A*, iodine value 2·6), leaving in solution in the ether 38·3 g. (*B*, iodine value 84·4).

There were thus obtained the following five groups of acids, each of which was methylated and fractionally distilled in the usual manner:

| Group | Weight (g.) | Weight (%) | Iodine value |
|---|---|---|---|
| *A* | 32·5 | 13·8 | 2·6 |
| *B* | 38·3 | 16·3 | 84·4 |
| *C* | 20·1 | 8·5 | 145·3 |
| *D* | 40·7 | 17·3 | 84·1 |
| *E* | 103·9 | 44·1 | 250·2 |

It is perhaps unnecessary to devote the space required for the presentation of the detailed ester-fractionation data (as in Table 1) and it is, therefore, proposed only to quote the fatty acid compositions finally obtained for each of the five groups of acids (%, w/w) and, therefrom, of the total fatty acids of the seal II blubber oil (Table 4).

Table 3. *Component acids in groups A, B, C and in the whole seal I blubber oil*

| Acid | Component acids in groups: *A* (13·8%)* (%)† | *B* (40·6%)* (%)† | *C* (45·6%)* (%)† | Fatty acids in the whole oil: (%, w/w) | (%, by mol.) | Mean unsaturation |
|---|---|---|---|---|---|---|
| Myristic | 9·7 | 1·6 | 0·9 | 2·4 | 2·9 | — |
| Palmitic | 65·0 | 3·2 | — | 10·3 | 11·1 | — |
| Stearic | 16·5 | 0·6 | — | 2·5 | 2·4 | — |
| Arachidic | 2·2 | — | — | 0·3 | 0·3 | — |
| Unsaturated $C_{14}$ | Trace | 1·9 | 4·7 | 2·9 | 3·6 | − 2·0 |
| Unsaturated $C_{16}$ | 1·6 | 28·5 | 30·4 | 25·7 | 27·8 | − 2·1 |
| Unsaturated $C_{18}$ | 3·3 | 51·8 | 24·3 | 32·6 | 31·8 | − 2·7 |
| Unsaturated $C_{20}$ | 1·2 | 12·3 | 15·2 | 12·1 | 10·8 | − 5·9 |
| Unsaturated $C_{22}$ | — | — | 24·4 | 11·2 | 9·3 | − 11·0 |
| Unsaponifiable | 0·5 | 0·1 | 0·1 | — | — | — |

* Group as % (w/w) of total oil. † Component acids as % (w/w) of group.

Table 4. *Component acids in groups A–E and in the whole seal II blubber oil*

| Acid | Component acids in groups: *A* (13·8%)* (%)† | *B* (16·3%)* (%)† | *C* (8·5%)* (%)† | *D* (17·3%)* (%)† | *E* (44·1%)* (%)† | Fatty acids in whole oil: (%, w/w) | (%, by mol.) |
|---|---|---|---|---|---|---|---|
| Myristic | 6·5 | 2·5 | 2·0 | 1·5 | 1·0 | 2·2 | 2·7 |
| Palmitic | 58·3 | 5·8 | 3·3 | 7·3 | — | 10·6 | 11·4 |
| Stearic | 28·7 | 2·1 | 1·4 | 0·1 | — | 4·4 | 4·3 |
| Arachidic | 2·3 | — | — | — | — | 0·3 | 0·3 |
| Unsaturated $C_{14}$ | Trace | 2·5 | 3·4 | 2·0 | 2·6 | 2·2 | 2·7 |
| Unsaturated $C_{16}$ | 1·0 | 14·1 | 32·1 | 28·1 | 24·2 | 20·8 | 22·7 |
| Unsaturated $C_{18}$ | 2·1 | 66·2 | 33·2 | 49·7 | 25·2 | 33·7 | 33·2 |
| Unsaturated $C_{20}$ | 0·7 | 6·5 | 22·7 | 11·2 | 19·4 | 13·6 | 12·4 |
| Unsaturated $C_{22}$ | — | — | 1·5 | — | 27·2 | 12·2 | 10·3 |
| Unsaponifiable | 0·4 | 0·3 | 0·4 | 0·1 | 0·4 | — | — |
| Mean unsaturation of: | | | | | | | |
| Unsaturated $C_{14}$ | − 2·0 | − 2·0 | − 2·0 | − 2·0 | − 2·0 | − 2·0 | — |
| Unsaturated $C_{16}$ | − 2·0 | − 2·0 | − 2·0 | − 2·0 | − 2·1 | − 2·1 | — |
| Unsaturated $C_{18}$ | − 2·0 | − 2·0 | − 2·5 | − 2·0 | − 3·0 | − 2·4 | — |
| Unsaturated $C_{20}$ | − 2·0 | − 5·0 | − 7·5 | − 4·5 | − 8·0 | − 7·2 | — |
| Unsaturated $C_{22}$ | — | — | − 11·0 | — | − 11·0 | − 11·0 | — |

* Group as % (w/w) of total oil. † Component acids as % (w/w) of group.

*Liver lipids*

*Seal I.* The moist liver tissue (338 g.) yielded to acetone a total quantity of 14·6 g. of lipids soluble in light petroleum, and contained 249 g. of water. The dry liver tissue thus contained 16·4% of lipids (4·3% on the moist tissue). These were dissolved in ten times their weight of acetone, and the solution left at 0° for some weeks, when 3·4 g. of sticky dark-brown material (phosphatides) were precipitated, leaving in solution 11·2 g. of glyceridic oil.

*Phosphatides* (crude) thus formed about 23% of the liver lipids. The amount available was too small to permit any examination of the fatty acids present. The material contained 4·7% P and 1·9% N (P:N ratio, 1:1·1).

About 77% of the seal liver lipids consisted of *glycerides* which, however, contained 18·5% of unsaponifiable matter. A portion (7·25 g.) of the liver-glyceride mixed fatty acids, from which unsaponifiable matter had been removed, was crystallized first from acetone at −60°, the deposited acids being further crystallized from ether at −40°. From the iodine values of the three fractions so obtained an approximate estimate of the saturated, monoethenoid and polyethenoid acids present was derived (assuming average iodine values of 90 and 350 respectively for the monoethenoid and polyethenoid material):

| | Weight (g.) | Weight (%) | Equivalent | Iodine value |
|---|---|---|---|---|
| (i) Insoluble in ether at −40° | 1·77 | 24·4 | 270·7 | 14·2 |
| (ii) Soluble in ether at −40° | 1·48 | 20·4 | 289·3 | 88·9 |
| (iii) Soluble in acetone at −60° | 4·00 | 55·2 | 312·1 | 267·0 |

from which is calculated:

| | Approximate component acids (expressed as % of total acids) | | |
|---|---|---|---|
| | Saturated | Monoethenoid | Polyethenoid |
| (i) | 20·5 | 3·9 | — |
| (ii) | 0·3 | 20·1 | — |
| (iii) | — | 17·7 | 37·5 |
| | 20·8 | 41·7 | 37·5 |

The component acids of the *seal I liver glycerides* consisted, therefore, of about 20% saturated acids and about 40% each of monoethenoid and polyethenoid fatty acids.

*Seal II.* The moist liver tissue (409 g.) yielded to acetone 43·6 g. of lipids soluble in light petroleum, i.e. 10·7% on the moist tissue. The moisture content of the latter is unfortunately uncertain, but the lipid content of the dry tissue must have been of the order of at least 50%. Thus the liver of the common seal II had a much higher fatty content than either that of the common seal I or that of the grey seal studied earlier (Hilditch & Pathak, 1947), and the subsequent examination showed that the increase in lipid content was wholly due to liver glycerides: the crude liver lipids (43·6 g.), after keeping in acetone solution at 0° for some weeks, deposited 2·3 g. of crude phosphatidic material, and left in solution 41·3 g. of glyceridic oil.

Crude phosphatides thus formed only 5% of the total liver lipids, and, again, were too small in amount for their fatty acids to be examined. The crude material contained 2·4% P and 1·0% N (P:N ratio, 1·06:1).

The crude glycerides contained 9·7% of unsaponifiable matter, so that the actual glyceride content of the liver lipids was about 86% (as compared with about 63% in the liver lipids of seal I). After removal of the unsaponifiable matter, the quantity of glyceridic mixed fatty acids (32·6 g.) available was sufficient for approximate analysis by ester fractionation (Table 5). The acids were first resolved into two fractions by crystallization from acetone at −60°:

| Group | Description | Weight (g.) | Weight (%) | Iodine value |
|---|---|---|---|---|
| *A* | Insoluble in acetone at −60° | 16·3 | 50·0 | 67·7 |
| *B* | Soluble in acetone at −60° | 16·3 | 50·0 | 299·4 |

Table 5. *Fractionation of methyl esters of seal II liver-glyceride fatty acids A and B*

| Fraction | Weight (g.) | Saponification equivalent | Iodine value |
|---|---|---|---|
| | Methyl esters of acids *A* | | |
| *A*1 | 2·15 | 269·3 | 15·5 |
| *A*2 | 1·77 | 276·2 | 23·6 |
| *A*3 | 2·58 | 283·9 | 36·3 |
| *A*4 | 3·21 | 298·2 | 60·1 |
| *A*5 | 2·84 | 299·5 | 72·5 |
| *A*6 | 3·20 | 316·3* | 90·6 |
| | 15·75 | | |
| | Methyl esters of acids *B* | | |
| *B*1 | 1·81 | 268·1 | 87·3 |
| *B*2 | 2·46 | 298·4 | 160·9 |
| *B*3 | 2·84 | 314·5 | 294·5 |
| *B*4 | 3·05 | 326·3 | 328·4 |
| *B*5 | 2·44 | 338·8 | 373·7 |
| *B*6 | 3·34 | 349·4* | 300·6 |
| | 15·94 | | |

* Equivalents of esters (freed from unsaponifiable matter): *A*6, 310·8; *B*6, 344·3.

The unsaturation of the homologous esters in group *B* was estimated to be as follows:

| Methyl esters of acids | Saponification equivalent | Iodine value | Mean unsaturation |
|---|---|---|---|
| $C_{16}$ | 268·0 | 94·8 | − 2·0 |
| $C_{18}$ | 294·5 | 151·0 | − 3·5 |
| $C_{20}$ | 318·0 | 319·2 | − 8·0 |
| $C_{22}$ | 343·0 | 407·3 | − 11·0 |

The fatty acid composition (%, w/w) of groups *A* and *B*, and thence those of the total fatty acids in the seal II liver glycerides, based on the ester-fractionation data in Table 5, are shown in Table 6.

At first sight it would appear that, as might be expected, the compositions of the fatty oils from the two common seals, taken at the same time in the same area, are not very different. Actually the only

Table 6. *Component acids in groups A and B, and in the whole seal II liver glycerides*

| | Component acids in groups | | Fatty acids in whole oil | |
|---|---|---|---|---|
| Acid | *A* (50·0%)* (%)† | *B* (50·0%)* (%)† | (%, w/w) | (%, by mol.) |
| Myristic | 0·3 | 0·2 | 0·2 | 0·3 |
| Palmitic | 22·1 | 0·7 | 11·4 | 12·9 |
| Stearic | 15·4 | — | 7·8 | 7·9 |
| Arachidic | 0·3 | — | 0·1 | 0·1 |
| Unsaturated $C_{16}$ | 6·8 (−2·0)‡ | 10·4 (− 2·0)‡ | 8·6 (− 2·0)‡ | 9·8 |
| Unsaturated $C_{18}$ | 40·4 (−2·0) | 15·1 (− 3·5) | 27·9 (− 2·4) | 28·7 |
| Unsaturated $C_{20}$ | 14·3 (−2·0) | 32·9 (− 8·0) | 23·7 (− 6·2) | 22·5 |
| Unsaturated $C_{22}$ | — | 40·4 (−11·0) | 20·3 (−11·0) | 17·8 |
| Unsaponifiable | 0·4 | 0·3 | — | — |

* Group as % (w/w) of total oil.
† Component acids as % (w/w) of group.
‡ Mean unsaturation.

## DISCUSSION

### *Seal blubber oils*

The data obtained in the course of the present work are most usefully considered in conjunction with the detailed component-acid figures which have been given for other seal blubber oils, namely, the grey seal (Hilditch & Pathak, 1947) and the mixed specimen of seal oil probably from Newfoundland or the north-eastern Atlantic seaboard (Burke & Jasperson, 1944). The percentages by weight of the component acids of all four oils are collected in Table 7.

significant difference is in the somewhat greater proportion of unsaturated (hexadecenoic) $C_{16}$ acids in the oil of seal I, which is offset by minor decreases in unsaturated $C_{18}$, $C_{20}$ and $C_{22}$ acids and in stearic acid as compared with the oil of seal II. The mean unsaturation of the $C_{20}$ acids in the oil of seal II is definitely greater than in the oil of seal I. Apart from these points, however, the two oils are not dissimilar: the chief component is the unsaturated $C_{18}$ group (about 33% of the total acids, and largely oleic acid) followed by about 21–25% of hexadecenoic acid; saturated acids, especially palmitic acid, are remarkably constant not only in these two oils, but in all four seal oils quoted in Table 7.

Table 7. *Component acids* (%, w/w) *of seal blubber oils*

| | Common seal I | Common seal II | Grey seal | Newfoundland (?) seal |
|---|---|---|---|---|
| *Iodine value of oil* | *140·0* | *145·4* | *162·2* | *135·8* |
| Acid: | | | | |
| Myristic | 2·4 | 2·2 | 3·7 | 5·1 |
| Palmitic | 10·3 | 10·6 | 10·5 | 10·7 |
| Stearic | 2·5 | 4·4 | 2·0 | 1·3 |
| Arachidic | 0·3 | 0·3 | — | 0·6 |
| Unsaturated $C_{14}$ | 2·9 | 2·2 | 1·6 | 1·8 |
| Unsaturated $C_{16}$ | 25·7 | 20·8 | 15·5 | 10·5 |
| Unsaturated $C_{18}$ | 32·6 | 33·7 | 30·8 | 39·6 |
| Unsaturated $C_{20}$ | 12·1 | 13·6 | 16·5 | 17·6 |
| Unsaturated $C_{22}$ | 11·2 | 12·2 | 18·1 | 10·6 |
| Unsaturated $C_{24}$ | — | — | 1·3 | 2·1 |
| Mean unsaturation of: | | | | |
| Unsaturated $C_{16}$ | − 2·1 | − 2·1 | − 2·2 | − 2·1 |
| Unsaturated $C_{18}$ | − 2·7 | − 2·4 | − 2·7 | − 2·4 |
| Unsaturated $C_{20}$ | − 5·9 | − 7·2 | − 5·7 | − 5·6 |
| Unsaturated $C_{22}$ | −11·0 | −11·0 | −10·6 | − 9·3 |
| Unsaturated $C_{24}$ | — | — | −11·0 | −10·9 |

It is only when comparison is made with the earlier results that it appears that the component acids of different seal blubber oils may vary to a considerable degree. The grey seal belongs to a different genus of the family, whilst the biological source of the Newfoundland oil cannot of course be given with any certainty. Whilst species differences may well account for at least part of the difference in composition between the blubber oils of the grey seal and the common seal, it is noteworthy that in these four oils we have a marked range of variation in some of the component acid groups, and that this is most evident in the hexadecenoic acid group, which lies between the extremes of about 10 and 26% of the total acids. In three of the four oils the unsaturated $C_{18}$ (mainly oleic) acid content is similar, but in the Newfoundland oil, with lowest unsaturated $C_{16}$ acid content, the percentage of the unsaturated $C_{18}$ group rises to nearly 40% of the total acids. Similarly, whilst the combined content of unsaturated $C_{20}$ and $C_{22}$ (and $C_{24}$) acids in the blubber oils of the common seal is about 23–26%, this rises in the grey seal oil to 36%; in the Newfoundland oil the unsaturated $C_{20}$ acid group is similarly high, but the unsaturated $C_{22}$ group is still at the level observed in the common seal oils. Apart from the $C_{20}$ acids in seal oil II (already mentioned), the mean unsaturation of the various series of acids appears to remain fairly constant in all the oils, irrespective of the variations in their proportion to which attention has just been drawn.

In the only other group of marine mammalian blubber oils—those of the Balaenidae—which have been studied to any extent, there are fairly well-defined differences in the proportions and average unsaturation of the different groups of component acids as between different genera and species of whales; but a number of independent specimens of Antarctic whale oil taken in different years show closely similar fatty acid compositions. So far as the few instances discussed above go, the conclusion may be hazarded that the blubber oils produced by different species of seal vary more widely in composition between themselves than those produced by different groups of the whale family. The present results may perhaps indicate further that the blubber fats of one and the same species of seal tend to exhibit more variation in composition than those from a single species of whale. This, again, may be connected with the diet of the seal, which is likely to be much more variable than that of the whale.

It is accordingly not easy to assign any very general characteristics to the component acids of seal blubber oils, except that their content of saturated acids is usually about 18% (10–11% palmitic acid), that unsaturated $C_{18}$ (mainly oleic) acid forms about 33% of the total acids, and that the rest is made up of unsaturated $C_{16}$ acids (about 15–25%) and polyethenoid acids of the $C_{20}$ and $C_{22}$ series (23–30%, mean unsaturation of the $C_{20}$ acids about $-5$ to $-6$ H, and of the $C_{22}$ acids $-10$ to $-11$ H).

*Seal liver lipids*

The liver tissue of the seal contains only a low proportion of lipids, which consist of glycerides and phosphatides in varying proportions. The liver of the common seal II apparently contained more lipid (glycerides) than the average, and in this instance we were able to make an ester-fractionation analysis of the liver-glyceride fatty acids. Comparative figures for the liver lipids of the common seal and of the grey seal are collected in Table 8.

Table 8. *Composition of seal liver lipids*

| | Common seal I | Common seal II | Grey seal |
|---|---|---|---|
| Total lipid content of liver (moist) (%) | 4·3 | 10·7 | 2·8 |
| Composition of liver lipids (%, w/w): | | | |
| Phosphatides (crude) | 23 | 5 | 29 |
| Glycerides | 63 | 86 | 50 |
| Unsaponifiable matter | 14 | 9 | 21 |
| Approximate composition of liver glyceride fatty acids (%, w/w): | | | |
| Saturated | 20 | 20 | 37 |
| Monoethenoid | 40 | 38 | 46 |
| Polyethenoid | 40 | 42 | 17 |

The composition of the liver lipids thus appears to be somewhat variable, with a ratio which perhaps in normal seal livers is of the order of two or three parts of glyceride to one of phosphatide. The component acids of the liver glycerides of the grey seal which we examined earlier (Hilditch & Pathak, 1947) were much less unsaturated than those of the corresponding blubber oil or of the liver glycerides of the common seal which we have now studied, and contained much less polyethenoid acids and nearly twice as much saturated acids as either of the latter. The detailed component acid figures for the liver and blubber glycerides of the common seal II are compared in Table 9.

Table 9. *Component acids of the liver and blubber glycerides of common seal II*

| Acid | Liver (%, w/w) | Blubber (%, w/w) |
|---|---|---|
| Myristic | 0·2 | 2·2 |
| Palmitic | 11·4 | 10·6 |
| Stearic | 7·8 | 4·4 |
| Arachidic | 0·1 | 0·3 |
| Unsaturated $C_{14}$ | — | 2·2 ( − 2·0)* |
| Unsaturated $C_{16}$ | 8·6 ( − 2·0)* | 20·8 ( − 2·1) |
| Unsaturated $C_{18}$ | 27·9 ( − 2·4) | 33·7 ( − 2·4) |
| Unsaturated $C_{20}$ | 23·7 ( − 6·2) | 13·6 ( − 7·2) |
| Unsaturated $C_{22}$ | 20·3 ( − 11·0) | 12·2 ( − 11·0) |

* Mean unsaturation.

The liver glycerides contained considerably less hexadecenoic acids, somewhat less oleic and polyethenoid $C_{18}$ acids, and considerably more polyethenoid $C_{20}$ and $C_{22}$ acids; the degree of unsaturation of the unsaturated components was similar in both liver and blubber glycerides. No comparable data are available for the liver and blubber glycerides of the whale or any other marine mammal, whilst similar comparisons in a number of fish species seem to indicate that no generalization can yet be made. Thus, in the sturgeon (Lovern, 1932*b*) and groper (Shorland & Hilditch, 1938) there is less fat in the liver than in the peritoneal cavity and pancreas (sturgeon) or the head (groper); but the component acids of liver and depot fats are similar in composition in the respective fish. This also obtains in the tunny (Lovern, 1936), in which the liver and flesh contain about the same proportion of fat. On the other hand, in halibut and turbot (Lovern, 1932*a*, 1937) the liver is the main fat depot, and the flesh contains but little fat: in both of these instances the unsaturated $C_{16}$ acid contents of the liver glycerides are much higher, and the amount of polyethenoid $C_{20}$ and $C_{22}$ acids much lower, than in the corresponding flesh fats. This lack of correlation stands in contrast to the fats of land animals in which (Hilditch & Shorland, 1937) the liver glycerides are distinguished by definitely lower contents of stearic acid, and higher contents of hexadecenoic and of polyethenoid acids of the $C_{20}$ and $C_{22}$ series, in comparison with the corresponding depot fats.

## SUMMARY

1. The composition of the blubber and liver lipids from two specimens of the common seal (*Phoca vitulina* L.) has been investigated.

2. The component acids of the blubber glycerides of the two animals were not dissimilar, but differed considerably from that of the grey seal (*Halichoerus grypus*). These differences are probably due partly to difference in species, but it also appears likely that differences in the food ingested by individual seals may be responsible to some extent for variations in the fat laid down.

3. The liver lipids of seals also appear to differ in amount and in their composition. One of the two common seal livers examined was exceptionally rich in glycerides as compared with phosphatides. Contrary to what was observed in the grey seal, the liver glycerides of the common seal are very similar in composition to its blubber oil.

4. So far, no correlation has been discernible between the composition of the liver glycerides and the glycerides of the flesh, head or other tissues of marine animals which may function as fat depots.

It is a pleasure once more to offer our thanks to Prof. A. N. Worden and to Dr L. Harrison Matthews for again providing us with interesting experimental material of authentic origin.

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# The Action of Trypsin on Insulin

By J. A. V. BUTLER, E. C. DODDS, D. M. P. PHILLIPS and J. M. L. STEPHEN
*The Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London*

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In previous studies (Butler, Dodds, Phillips & Stephen, 1948) it was found that the action of trypsin on insulin is comparatively slight. It appeared likely that the slow action observed was due to a small proportion of chymotrypsin (about 0·5%) known to be present in the trypsin, and it was doubtful if there was any residual effect which might be ascribed to trypsin itself. In some further experiments with the same materials it was found that the extent of the action was rather variable, and appeared to depend on several factors, e.g. the method of preparing the insulin solution, the age of the solution at the time of enzyme addition. The possibility that trypsin might cause a slight hydrolysis of insulin without decreasing its potency has led us to re-examine its action, using eventually

a purified trypsin kindly given us by Drs J. H. Northrop and M. Kunitz, and a sample of insulin which had been several times recrystallized.

## EXPERIMENTAL AND RESULTS

### *The effect of trypsin on insulin solutions prepared in several ways*

These experiments were carried out as described in a previous paper (Butler *et al.* 1948), using the same trypsin, and an insulin concentration of 5 mg./ml. The insulin solutions were made up as indicated in Table 1. The extent of hydrolysis certainly varied with the method of preparation of the solution, but in most cases was comparatively small in the first 24 hr., and only became considerable after that period. This was probably due to the slow action of the chymotrypsin present on the insulin, which converts the latter into products upon which trypsin can act. Initial treatment with alkali, apparently, makes the insulin more susceptible to attack under these conditions.

### *Action of purified trypsin*

The action of the purified enzyme on the commercial insulin was found, as might be expected, to be appreciably less especially in long periods than that of the trypsin used above, but a real residual action still remained. Table 2 shows the results obtained in two distinct experiments. In both cases there is an increase of non-protein nitrogen (N.P.N.) amounting to about 7% in 1 day and 12% in 2–3 days, and the amino N increases at the same time to about 10%, which would signify a quite appreciable splitting of about 6 peptide bonds/insulin unit of 12,000. At the end of the experiments, the pH of the solutions was adjusted to 5·4 and the precipitates which formed were filtered off and dried. Their insulin potency, kindly determined for us by Boots Pure Drug Co. Ltd., was 56–59% of that of the original insulin. It is evident that in the long-continued action of trypsin there had been considerable loss of potency.

### *Action of purified trypsin on recrystallized insulin*

There remains some doubt whether the non-protein N formed comes from insulin or extraneous substances. The insulin used was Boots zinc insulin, potency 22·8 units/mg. This was found to give a single sharp peak in the electrophoretic diagram. It was recrystallized four times, the first three by the method of Romans, Scott & Fisher (1940), and the last time by dissolving in dilute acetic acid and adding NaOH to pH 4·5. It should be pointed out that, according to a recent paper by Lens (1948), this procedure may result in the formation of some denatured material. The results obtained by digesting this insulin (5 mg./ml.) with $135 \times 10^{-4}$ units of the purified trypsin in 100 ml. are shown in Table 3 A. A similar control experiment without any trypsin present is shown in Table 3 B. It is evident that the change in N.P.N. in the presence of trypsin was not much greater than that in a similar buffer containing no trypsin. The insulin, however, lost a rather greater amount of potency in the presence of the trypsin than in its absence. The potency of the recovered material from the trypsin solution after 72 hr. was greater than that after 48 hr.; this may be connected with the fact that a smaller proportion of the insulin was actually

Table 1. *Tryptic action on insulin solutions prepared in various ways*

(Non-protein N (N.P.N.) expressed as % of total N.)

| *A* | | *B* | | *C* | | *D* | | *E* | |
|---|---|---|---|---|---|---|---|---|---|
| Time (hr.) | N.P.N (%) | Time (hr.) | N.P.N. (%) | Time (hr.) | N.P.N. (%) | Time (hr.) | N.P.N. (%) | Time (hr.) | N.P.N. (%) |
| 0 | — | 0 | 7·6 | 0 | 7·3 | 0 | 9·7 | 0 | 11·6 |
| 17·5 | 9·7 | 23 | 14·7 | 18 | 10·9 | 23 | 13·6 | 18 | 17·5 |
| 44·5 | 19·2 | 46 | 18·0 | 42 | 44·7 | 48 | 45·6 | 42 | 23·2 |

*A*. Insulin mixed with phosphate buffer, pH 7·6, before adding enough alkali to bring it into solution. Trypsin added at once.
*B*. Same as *A*, but trypsin added 70 hr. after making up solution.
*C*. Insulin dissolved in 0·02 N-NaOH and same quantity of phosphate buffer then added. Trypsin added at once.
*D*. Same as *C*, but trypsin added 21 hr. after making up solution.
*E*. Same as *A*.

Table 2. *Action of purified trypsin on insulin*

(Digest contains 5 mg. insulin/ml. in M/24 phosphate buffer, pH 8·2.)

| Trypsin: $44 \times 10^{-4}$ units in 30 ml. | | | Trypsin: $72 \times 10^{-4}$ units in 50 ml. | | |
|---|---|---|---|---|---|
| Time (hr.) | N.P.N. (%) | Amino N (%) | Time (hr.) | N.P.N. (%) | Amino N (%) |
| 0 | 5·5 | 4·4 | 0 | 6·6 | 4·4 |
| 3 | 5·7 | — | 23 | 14·4 | 9·3 |
| 21 | 12·5 | — | 47 | 18·9 | 9·5 |
| 44·5 | 12·5 | — | | | |
| 68·5 | 17·2 | 10·0 | | | |
| N precipitated at pH 5·4, 82% | | | N precipitated at pH 5·4, 84·5% | | |
| Potency of precipitate 13·4 units/mg. | | | Potency of precipitate 12·7 units/mg. | | |

Table 3. *Comparison of changes in digest with those in buffer alone*

A. Action of purified trypsin on 4 times recrystallized insulin (25°)

| Time (hr.) | 0 | 48 | 72 |
|---|---|---|---|
| N (% of T.N.)* in trichloroacetic filtrate. (N.P.N.) | 4·0 | 7·6 | 11·1 |
| N (% of T.N.) in isoelectric filtrate | — | 6·4 | 11·5 |
| Amino N (% of T.N.) in recovered insulin | 4·4 | 6·3 | 5·8 |
| Amide N (% of T.N.) in recovered insulin | 9·9 | 8·4 | 9·0 |
| Mean potency of precipitate (units/mg.) | 22·96 | 15·6 | 18·7 |
| Limits of error, $P=0·95$ | 21·01–25·09 | 13·69–17·71 | 15·89–22·13 |

B. Change of insulin with time in buffer solution, pH 8·4 (25°)

| Time (hr.) | 0 | 24 | 48 | 72 |
|---|---|---|---|---|
| (*a*) N (% of T.N.) in trichloroacetic filtrate. (N.P.N.) | 4·9 | 6·2 | 8·6 | 10·0 |
| Potency of precipitate (units/mg.) | 22·9 | — | — | 20·6 |
| Limits of error, $P=0·95$ | — | — | — | 18·5–22·9 |
| (*b*) N (% of T.N.) in isoelectric filtrate | 0·0 | 2·2 | 3·2 | 4·8 |
| Amide N (% of T.N.) in recovered insulin | 9·3 | 9·4 | 9·5 | 8·3 |

* T.N. = Total nitrogen.

recovered in the former case. There was a slight increase in the percentage of amino N in the recovered insulin from trypsin, which may indicate the breakage of one or two peptide bonds. The results shown in lines (*a*) and (*b*), Table 3B, were obtained in separate experiments. The N.P.N. values obtained in this experiment seem rather high; they are quoted because the potency of the insulin recovered from the buffer after 72 hr. was also determined. A re-determination of the N.P.N. in the trichloroacetic acid filtrate after 72 hr. (exp. *b*) gave a value only about 3% greater than the N in the isoelectric filtrate. It is evidently difficult to reproduce the N.P.N. formed in buffer solutions after long periods of time.

Dr K. O. Pedersen of Uppsala has kindly examined in the ultracentrifuge the products of trypsin action dissolved in a buffer solution containing $Na_2HPO_4$ (0·10 M) and $NaH_2PO_4$ (0·05 M). The sedimentation constant of the bulk of the material was found to be the same as that of untreated insulin (Pedersen, 1948), but a spreading of the sedimentation curves indicated that the material had become less homogeneous. The possibility that the degradation of insulin in the buffer solution was due to the contamination of the insulin by a proteolytic enzyme was considered. No evidence of the existence of the latter could be obtained when insulin was added to the haemoglobin substrates suggested for the assay of trypsin and pepsin by Anson (1938), but the concentration necessary to produce the effect with insulin could be too small to detect by the latter method.

Since trypsin exerts an amidase activity on certain amides (Bergmann, Fruton & Pollock, 1939; Schwert, Neurath, Kaufman & Snoke, 1948), and since insulin contains 8·9% of its nitrogen as amide N (Chibnall, 1942), we have also attempted to find if any of the latter is liberated by trypsin. The amide N was determined in a conventional manner (see Sanger, 1945), but no significant diminution was observed in trypsin-treated insulin.

*Purification of insulin by treatment with trypsin*

If, unlike other proteins, insulin is resistant to trypsin it should be possible to purify it to some extent, at least, by tryptic digestion. A crude insulin preparation, kindly given to us by Boots Pure Drug Co. Ltd., had a potency of 8·2 units/mg. This material (0·5 g.) was dissolved in 85 ml. of phosphate buffer, pH 7·8, and treated with crystalline trypsin ($500 \times 10^{-4}$ units) for 19 hr. at 25°. The pH was then adjusted to 5·2 with 0·1 N-HCl and the precipitate filtered off (dry wt. 59 mg.). Its potency, as determined by Boots, was 17·0 units/mg., i.e. the potency of the insulin had been doubled, but there was a considerable loss, since only 1000 units were recovered from an initial 4000. It is possible that purified trypsin would give a more satisfactory recovery, and that trypsin action may be a useful step in the purification of trypsin-resistant proteins.

## DISCUSSION

Every increase in the purity of the enzyme preparation and of the insulin has resulted in a decrease of the action observed, and under the best conditions the change produced is slight. The increase in non-protein nitrogen in the presence of trypsin is of the same order as that in the buffer alone. There is, however, a slight increase in the free amino nitrogen of the recovered insulin and the potency of the recovered material is certainly appreciably less. It is therefore possible that the trypsin breaks one or two bonds in the insulin molecule, per unit of mol. wt. 12,000, without any non-protein peptide being liberated. The ultracentrifuge also shows a change in the nature of the recovered insulin. Although its mean sedimentation constant is unchanged, there is a greater spread of sedimentation rate, indicating an increased heterogeneity of the material. This might be due to a slight denaturation affecting the shape of the molecules and the way in which the submolecules aggregate.

## SUMMARY

1. The action of trypsin on insulin has been re-examined. Purified trypsin has less action than the trypsin originally used.

2. Using four times recrystallized insulin and purified trypsin, the non-protein nitrogen formed is not much greater than that produced in the buffer

alone, but in the presence of trypsin the physiological potency is diminished to a somewhat greater extent than in its absence. Ultracentrifugal examination of the recovered insulin shows it to have become less homogeneous than the original.

3. The action of trypsin on insulin is thus slight, but the possibility of the hydrolysis of one or two peptide bonds, with a diminution of potency of 10–20%, is not excluded.

One of us (J.A.V.B.) holds the Courtauld Research Fellowship in this Institute. In addition our thanks are due to Courtaulds Ltd. for a grant in aid of this investigation; to Imperial Chemical Industries Ltd. for financial assistance; to Sir J. Drummond, F.R.S., and to Dr W. A. Broom of Boots Pure Drug Co. Ltd., for assays of the insulin preparations; to Drs J. H. Northrop and M. Kunitz for purified trypsin; to Dr K. O. Pedersen for the ultracentrifugal examination of some of the products; and to Mrs I. D. R. Goodwin for technical assistance.

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# Studies in the Biochemistry of Micro-organisms

## 80. THE COLOURING MATTERS OF *PENICILLIUM ISLANDICUM* SOPP. PART 1. 1:4:5-TRIHYDROXY-2-METHYLANTHRAQUINONE

BY B. H. HOWARD AND H. RAISTRICK
*Department of Biochemistry, London School of Hygiene and Tropical Medicine, University of London*

(*Received* 30 *July* 1948)

*Penicillium islandicum* Sopp is a species included by Thom (1930) in the Funiculose series of the division of the Penicillia known as the Biverticillata-symmetrica. The species was first described and named by Sopp (1912). He isolated it in Norway from a mouldy specimen of skyr received from Reykjavik, Iceland. Skyr* is a bacterially soured milk peculiar to Iceland, and is somewhat similar in nature to Bulgarian yogurt. A number of other strains have been isolated subsequently in different parts of the world. Colonies of *P. islandicum* are characterized by their three-coloured appearance, 'a green conidial zone near the margin and progressively overgrown with red orange to red hyphae in the central areas, reverse at first orange to sordid yellow orange shades, later becoming rich red shades' (Thom, 1930). We are engaged in a study of this complex mixture of colouring matters, and it is the purpose of the present communication to describe the isolation of one of them and its identification as the hitherto undescribed 1:4:5-*trihydroxy-2-methylanthraquinone*.

* Thom (1930, p. 466), in a description of *P. islandicum* Sopp, says 'species found on the Island of Skyr'. Sopp's (1912, p. 162) original description states 'Der Pilz wurde auf isländischem Skyr gefunden'.

The new colouring matter, which was isolated by solvent extraction from five different strains of *P. islandicum* grown on Czapek-Dox 5% glucose solution, was obtained in yields of 3% of the dried mycelium of strain 1036. The total yield of the fat-free complex mixture of colouring matters was rather remarkable. It amounted to 20% of the dried mycelium. The nature of the other colouring matters is being investigated.

The new colouring matter forms large, dark red, lustrous plates or leaflets, m.p. 218°. It has the molecular formula $C_{15}H_{10}O_5$, contains one methyl group attached to carbon, no methoxyl group, and forms a triacetate, m.p. 208°, and a trimethyl ether, m.p. 161°, mol. wt. 314, 320. It is not soluble in aqueous sodium carbonate, but dissolves readily in sodium hydroxide giving a deep violet solution. Its solution in cold concentrated sulphuric acid is bright purple red in colour with a fiery red fluorescence. Its general behaviour is that of a polyhydroxyanthraquinone.

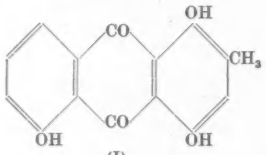
Its molecular structure as 1:4:5-trihydroxy-2-methylanthraquinone was established as follows. Since its molecular formula is $C_{15}H_{10}O_5$, and it contains one methyl group and three hydroxyl groups (formation of a triacetate and a trimethyl ether), it

appeared probable that it was a trihydroxymethylanthraquinone. This hypothesis was confirmed by converting it, by mild oxidation with manganese dioxide and concentrated sulphuric acid, into the known compound, cynodontin (V). Cynodontin, 1:4:5:8-tetrahydroxy-2-methylanthraquinone, was first isolated by Raistrick, Robinson & Todd (1933*a*) from laboratory cultures of *Helminthosporium cynodontis* Marignoni and *H. euchlaenae* Zimmermann and later by the same workers (Raistrick, Robinson & Todd, 1934) from *H. avenae* Eidam. They established its constitution as (V) and this was confirmed by its synthesis by Anslow & Raistrick (1940*b*). Hence it follows that the *Penicillium islandicum* colouring matter must be one of the four possible 1:4:5-trihydroxy-$\beta$-methylanthraquinones of structures (I), (II), (III), (IV). Of these, (III) and (IV) are known compounds.

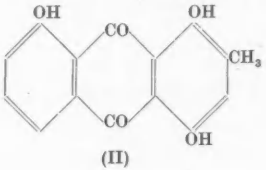
comparison of the *Penicillium islandicum* colouring matter with helminthosporin left no doubt that they were not identical, although they give colour reactions with sodium hydroxide and cold concentrated sulphuric acid which are almost indistinguishable from each other.

Compound (IV) has been synthesized by two different methods. Graves & Adams (1923) state that, on heating, (IV) sublimes at 250–260°. Keimatsu & Hirano (1930) give the m.p. of (IV) as 253–254°, its triacetate as 215° and its trimethyl ether as 206·5–207°. The corresponding melting points for the *P. islandicum* colouring matter are 218°, 208° and 161°. It is thus clear that the *P. islandicum* colouring matter is not identical with (IV).

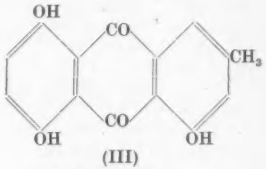
Hence it follows that the *P. islandicum* colouring matter must have either structure (I) or (II). This conclusion was confirmed and a choice made



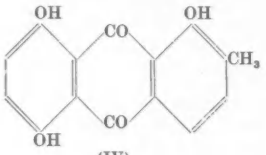

(I)
*P. islandicum* product



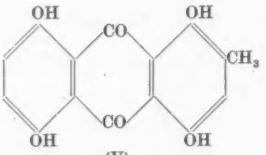

(II)



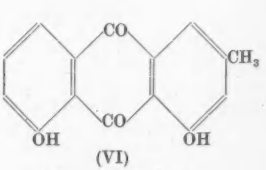

(III)
Helminthosporin



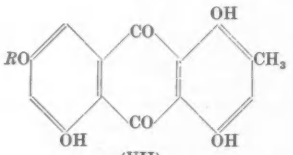

(IV)



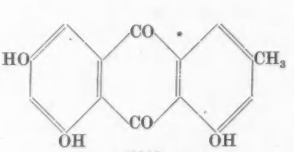

(V)
Cynodontin



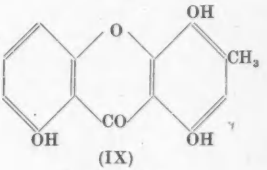

(VI)
Chrysophanic acid




(VII)
$R$ = H, Catenarin
$R$ = $CH_3$, Erythroglaucin




(VIII)
*Frangula* emodin




(IX)
Ravenelin

Compound (III) is helminthosporin which was isolated from cultures of *Helminthosporium gramineum* Rabenhorst by Charles, Raistrick, Robinson & Todd (1933); also from *H. cynodontis* Marignoni (Raistrick *et al.* 1933*a*); and *H. catenarium* Drechsler and *H. tritici-vulgaris* Nisikado by Raistrick *et al.* (1934). Its molecular constitution, (III), was established by analytical means by Charles *et al.* (1933), and this was confirmed by its synthesis by Graves & Adams (1923) and by Raistrick, Robinson & Todd (1933*b*). Direct between (I) and (II) by the following method. The *P. islandicum* colouring matter was reduced with concentrated hydriodic acid and red phosphorus in glacial acetic acid. The resulting anthranol, on oxidation with chromic acid in acetic acid solution, yielded chrysophanic acid (chrysophanol), the structure of which, (VI), was established by synthesis by Naylor & Gardner (1931). Of the structures (I), (II), (III) and (IV) only those which could give chrysophanic acid, (VI), by removal of any one hydroxyl group are (I) and (III). Compound (III)

is helminthosporin. Hence it follows that the *P. islandicum* colouring matter must be (I), i.e. 1:4:5-trihydroxy-2-methylanthraquinone.

It is interesting to note that the *P. islandicum* colouring matter is closely related structurally, not only to the mould colouring matters helminthosporin and cynodontin, but also to catenarin (VII, $R=H$) and erythroglaucin (VII, $R=CH_3$), which are respectively the 7-hydroxy and 7-methoxy derivatives of the *P. islandicum* colouring matter. Catenarin was isolated from laboratory cultures of *Helminthosporium catenarium* Drechsler, *H. velutinum* Link and *H. tritici-vulgaris* Nisikado by Raistrick *et al.* (1934). Its molecular constitution (VII) was established by Anslow & Raistrick (1940*a*), and this was confirmed by synthesis by the same investigators (Anslow & Raistrick, 1941). Erythroglaucin was isolated by Ashley, Raistrick & Richards (1939) from a number of species in the *Aspergillus glaucus* series, and its molecular constitution was established by Anslow & Raistrick (1940*a*). They also showed that, on reduction with hydriodic acid and red phosphorus in glacial acetic acid followed by oxidation of the resulting anthranol with chromic acid, catenarin (VII) yields *Frangula* emodin (VIII). We have stated previously that the *Penicillium islandicum* colouring matter (I), on similar treatment, yields chrysophanic acid (VI). It will be noted that in each case it is the hydroxyl group in the ortho position to the methyl group which is removed.

A further example of the close structural relationship between many mould colouring matters is afforded by ravenelin. This substance, 1:4:8-trihydroxy-3-methylxanthone (IX), was isolated from *Helminthosporium ravenelii* Curtis and *H. turcicum* Passerini, and its molecular constitution established by Raistrick, Robinson & White (1936). Its structural relationship to the *Penicillium islandicum* colouring matter (I) is obvious.

Two trihydroxymethylanthraquinones of unknown molecular constitution having properties similar to those of 1:4:5-trihydroxy-2-methylanthraquinone have been described. Nataloemodin (m.p. 214–215°; triacetate, m.p. 204°) was obtained by Léger (1902, 1905) by degradation of nataloin. He found that nataloin prepared from Natal aloes, on treatment with alkaline sodium peroxide, yielded nataloemodin monomethyl ether, which on demethylation gave nataloemodin. Through the courtesy of Dr T. E. Wallis, Curator of the Museum of the Pharmaceutical Society, London, we received a specimen of nataloemodin monomethyl ether made by Léger. We demethylated this substance with hydrobromic acid in glacial acetic acid solution. The resulting nataloemodin, m.p. 216°, has properties quite different from 1:4:5-trihydroxy-2-methylanthraquinone, and is clearly not identical with it. Funiculosin ($C_{15}H_{10}O_5$; m.p. 218°; triacetate, m.p. 205°; methoxy, nil) was isolated by Igaraci (1939) from laboratory cultures of *P. funiculosum* Thom. Its molecular constitution was not established, though Igaraci expressed the opinion that it is either a trihydroxymethylanthraquinone or a dihydroxyhydroxymethylanthraquinone. This view is, however, difficult to reconcile with Igaraci's finding that funiculosin gives anthracene and not, as would be expected, a methylanthracene on zinc dust distillation. The identity of the anthracene was established by melting point, mixed melting point and analysis, and confirmed by conversion into anthraquinone. However, since *P. islandicum* Sopp and *P. funiculosum* Thom are regarded by Thom (1930) as closely related species, both being included in the *P. funiculosum* series of the Biverticillata-symmetrica, we prefer to regard the question of the identity of funiculosin with the *P. islandicum* colouring matter as an open one for the present. For this reason we prefer in the meantime not to give the *P. islandicum* colouring matter a trivial name.

## EXPERIMENTAL

### *Cultures*

Six different strains of *P. islandicum* Sopp have been examined in the course of this work. They were all received, from the Thom-Raper collection, from Dr Kenneth B. Raper, Northern Regional Research Laboratory (N.R.R.L.), U.S. Department of Agriculture, Peoria, Illinois, U.S.A. Their history, kindly supplied by Dr Raper, is as follows.

1. Strain N.R.R.L. 1036: received originally in 1922 from F. M. Putterill, Cape Town, South Africa.

2. Strain N.R.R.L. 1037: received originally in 1924 from Dr Nakata. Isolated in Japan among moulds commonly found in the citrus industry.

3. Strain N.R.R.L. 1038: received originally in 1927 from Dr W. Schwartz from Germany.

4. Strain N.R.R.L. 1175: received originally in 1940 from Dr G. A. Ledingham, Ottawa, Canada.

5. Strain N.R.R.L. 1177: received originally in 1940 from Dr G. A. Ledingham, Ottawa, Canada. This strain was isolated from a sample of wet flour.

6. Strain N.R.R.L. 2115: received originally in 1942 from Dr Franz Lozet, Leopoldville, Belgian Congo.

Strain N.R.R.L. 1036 was received by us in October 1943; the other strains in December 1947.

1:4:5-Trihydroxy-2-methylanthraquinone was isolated from all six strains with the exception of Strain N.R.R.L. 1175 which, although morphologically a typical strain, appears to be biochemically atypical. Strain N.R.R.L. 1036 was used for the bulk preparation of 1:4:5-trihydroxy-2-methylanthraquinone.

### *Cultural characteristics of, and pigment formation by, different strains of* Penicillium islandicum

Quantities of 350 ml. of Czapek-Dox solution (glucose, 50·0 g.; $NaNO_3$, 2·0 g.; $KH_2PO_4$, 1·0 g.; KCl, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g.; distilled water,

1 l.) were distributed in each of eighteen 1 l. conical flasks, plugged with cotton wool and sterilized. A batch of three flasks was inoculated with one of each of the six strains of *P. islandicum* N.R.R.L. 1036, 1037, 1038, 1175, 1177 and 2115. One flask of each strain was harvested after 7, 14 and 21 days' incubation at 24° in the dark. The mycelium was separated by filtration, washed with water, and dried *in vacuo*.

*Examination of the culture filtrates*

The culture filtrates of all strains except 1175 were similar in appearance. They were all orange-red in colour but varied somewhat in intensity, and filtered somewhat slowly. There remained on the filter paper a reddish deposit varying in amount with the different strains, and containing many microcrystalline orange-red clusters of needles. On the other hand, strain 1175 gave an orange-yellow culture filtrate with no sign of red colouring matter. The pH (Table 1, col. 3) varied within narrow limits for all strains.

*Antibiotic activity.* The antibiotic activity (Table 1, col. 4) was measured by the serial dilution method against *Staphylococcus aureus*, Oxford H strain. All strains except 1175 at some period of growth totally inhibited the growth of *Staph. aureus* at dilutions of 1:20 to 1:80.

Table 1. *Cultural characteristics of six strains of* Penicillium islandicum *Sopp, and yields of colouring matters formed by them*

| Catalogue no. (N.R.R.L.) (1) | Incubation period (days) (2) | pH after incubation (3) | Antibiotic activity (4) | Wt. of mycelium (g.) (5) | Mycelium total wt. (g.) (6) | 'Fat' (g.) (7) | $Na_2CO_3$-soluble pigment (g.) (8) | $Na_2CO_3$-insoluble pigment (g.) (9) | Crystalline 1:4:5-trihydroxy-2-methylanthraquinone (g.) (10) |
|---|---|---|---|---|---|---|---|---|---|
| 1036 | 7 | 3·6 | 1:20 | 2·13 | 10·34 | 0·87 | 1·41 | 1·19 | 0·20 |
| | 14 | 3·8 | Nil | 3·76 | | | | | |
| | 21 | 4·3 | Nil | 4·45 | | | | | |
| 1037 | 7 | 3·6 | 1:80 | 1·99 | 9·84 | 0·76 | 0·60 | 0·65 | 0·18 |
| | 14 | 3·8 | 1:80 | 3·96 | | | | | |
| | 21 | 4·2 | 1:20 | 3·89 | | | | | |
| 1038 | 7 | 3·6 | 1:20 | 1·56 | 7·49 | 0·41 | 0·31 | 0·42 | 0·04 |
| | 14 | 3·9 | 1:20 | 2·91 | | | | | |
| | 21 | 4·4 | 1:40 | 3·02 | | | | | |
| 1175 | 7 | 3·7 | Nil | 2·33 | 9·90 | 0·50 | 0·77 | 0·57 | Nil |
| | 14 | 3·7 | Nil | 3·62 | | | | | |
| | 21 | 4·0 | Nil | 3·95 | | | | | |
| 1177 | 7 | 3·6 | 1:20 | 1·76 | 9·46 | 1·06 | 0·82 | 1·16 | 0·09 |
| | 14 | 4·1 | 1:20 | 3·55 | | | | | |
| | 21 | 4·4 | Nil | 4·15 | | | | | |
| 2115 | 7 | 3·6 | 1:80 | 1·82 | 8·46 | 0·69 | 0·47 | 0·49 | 0·05 |
| | 14 | 3·7 | 1:40 | 3·26 | | | | | |
| | 21 | 3·8 | 1:40 | 3·38 | | | | | |

$FeCl_3$ *reaction.* Addition of aqueous M/6-$FeCl_3$ to the culture filtrate gave with all strains a heavy, yellow to brown, amorphous precipitate, which usually appeared after 15 min. and always appeared on standing overnight. To 300 ml. of culture filtrate from each strain after 7 days' growth were added 30 ml. of aqueous M/6-$FeCl_3$. The resulting precipitates were separated by centrifuging, washed with water, and dissolved in 2 N-HCl (8 ml.), giving yellow to brownish red solutions. Addition of ethanol (50 ml.) gave white to very pale yellow amorphous precipitates which were washed with ethanol and finally with ether, and dried. The following weights were obtained: Strain 1036, 0·36 g.; 1037, 0·58 g.; 1038, 0·41 g.; 1175, 0·17 g.; 1177, 0·30 g.; 2115, 0·76 g. The nature of this material has not yet been fully investigated, but it appears to be a phosphorylated polysaccharide.

*Bromine reaction.* Addition of saturated bromine water (2 ml.) to 10 ml. of culture filtrate gave a characteristic reaction with all strains and at all stages of growth. The mixture remained clear for some time, but on standing overnight there was formed a heavy, yellow to orange, gelatinous precipitate.

*Examination of mycelia*

The appearance of the mycelia of strains 1036, 1037, 1038, 1177 and 2115 varied slightly, particularly in the early stages of growth, but in the later stages the strains were almost indistinguishable from each other. They all developed reddish drops on the surface of the mycelium which, at the end of the incubation period, consisted mainly of reddish brown to brownish red growth with small areas of green growth. The reverse of the mycelium was in all cases a dark reddish brown to brownish red. The colour of the mycelium of strain 1175 was much lighter, being yellow to orange with green areas and with a yellow to brown reverse.

Small portions of the mycelia were pressed between layers of filter paper, and then part was immersed in cold conc. $H_2SO_4$, and part in aqueous N-NaOH. The mycelia of strains 1036, 1037, 1177 and 2115 developed deep to very deep violet colours with conc. $H_2SO_4$ at all stages of growth. Strain 1038 gave a brownish red colour at 7 days, becoming deep reddish violet after 14 days, and deep violet after 21 days. With strain 1175 the corresponding colours were pale brown, dark reddish violet, and brownish red. With N-NaOH, strains 1036, 1037, 1177 and 2115 developed a

series of green shades of colour, varying from clear emerald-green through olive-green to bluish green. The colours with 1038 were shades of violet, and with 1175 reddish violet to a clear purple red.

The dried mycelia, after 7, 14 and 21 days' growth of each strain, were combined and powdered. (Weights, see Table 1, col. 6.) The powder was extracted exhaustively with chloroform in a Soxhlet apparatus. A dark red extract was obtained in all cases except strain 1175 which gave a brown-yellow extract. The solvent was removed by distillation *in vacuo* and the residual solid was extracted with light petroleum. The pale coloured light petroleum extract, on evaporation to dryness, left a red coloured 'fat'. (Weights, see Table 1, col. 7.)

The material insoluble in light petroleum was dissolved in chloroform (1 l.) and extracted with aqueous N-$Na_2CO_3$. After centrifugal separation the $Na_2CO_3$ extracts were acidified. The resulting red precipitate was filtered, washed, and dried. (Weights, see Table 1, col. 8.)

The residual chloroform solutions were evaporated to low bulk. In the cases of strains 1036 and 1037 crystals of crude 1:4:5-trihydroxy-2-methylanthraquinone (weights, 0·15 g., m.p. 214–215°, and 0·15 g., m.p. 212–215°, respectively) separated. With the other four strains no crystals separated. The mother liquors from 1036 and 1037 and the solutions from 1038, 1175, 1177 and 2115 were dried (total weight of $Na_2CO_3$ insoluble colouring matter, Table 1, col. 9). The residues, except strain 1175, were sublimed in a high vacuum. A red sublimate was obtained in each case at 150–200°. The sublimates were crystallized once from glacial acetic acid, and the total weights of crystalline material isolated are given in Table 1, col. 10. The different crystalline preparations all melted within the range 216–217·5°, and none of them depressed the m.p. of 1:4:5-trihydroxy-2-methylanthraquinone on admixture with a pure specimen of it, m.p. 218°.

### *Preparation of* 1:4:5-*trihydroxy*-2-*methylanthraquinone from* Penicillium islandicum *Sopp, strain N.R.R.L.* 1036

Czapek-Dox 5% glucose solution was distributed, in 350 ml. quantities, into a number of 1 l. conical flasks, plugged with cotton wool and sterilized by steaming for 0·5 hr. on each of 3 consecutive days. In typical preparations 50 such flasks were inoculated with a spore suspension of *P. islandicum*, strain N.R.R.L. 1036, and incubated in the dark at 24° for 19–21 days. At the end of this period the dark red mycelial mat was separated by filtration, washed with water, dried *in vacuo*, and ground in a coffee mill, yielding 201 g. of a brownish red powder. Treatment of the culture filtrate, which contained 0·1% of residual glucose, will form the subject of a future communication. The ground mycelium was freed from fat by boiling under reflux with three separate lots of 1 l. of light petroleum (b.p. 40–60°). The light orange-coloured combined extracts were thoroughly extracted with aqueous 0·5 N-NaOH which, on acidification, gave 0·76 g. of fat-contaminated colouring matter. The colourless residual light petroleum yielded 10·7 g. of 'fat' on removal of the solvent.

The colouring matters were extracted from the fat-free powdered mycelium by boiling under reflux for 1–2 hr. each time with seven successive lots of 1 l. of chloroform. The chloroform solutions were all brownish red to orange-red in colour. A rough fractionation of the mixture of colouring matters was effected by thorough extraction of their chloroform solutions with aqueous $Na_2CO_3$ in which 1:4:5-trihydroxy-2-methylanthraquinone is not soluble. N-$Na_2CO_3$ was used for the earlier and more concentrated chloroform solutions and 0·25 N for the later ones. The somewhat emulsified mixtures were separated by centrifuging and the $Na_2CO_3$ extracts, varying in colour from brown with the earlier extracts to pure green with the later ones, were quickly acidified with HCl. The resulting dark red precipitates were separated by filtration, thoroughly washed with water, and dried. A dark red powder, total weight 19·15 g. was thus obtained and the fractionation of this material is at present under investigation.

Isolation of 1:4:5-trihydroxy-2-methylanthraquinone from the chloroform solutions, free from $Na_2CO_3$-soluble substances, may be accomplished by either of the following methods. (*a*) The chloroform solutions were washed with water, filtered and evaporated to crystallization point. Second crops were obtained by further evaporation and crystallization. A total yield of 6·19 g. of crude 1:4:5-trihydroxy-2-methylanthraquinone was obtained. The crude product was purified by repeated recrystallization from chloroform or glacial acetic acid yielding shining red plates, m.p. 218°. On removal of the solvent from the original chloroform mother liquors there remained 18·82 g. of a mixture of colouring matters. (*b*) The chloroform solutions were combined and thoroughly extracted with aqueous 0·5 N-NaOH. This treatment removed the remainder of the colouring matters leaving an almost colourless chloroform solution. Acidification of the bluish purple NaOH extract with HCl yielded a red amorphous solid which was separated by filtration, washed with water, and dried. This was heated in small portions in a high vacuum when 1:4:5-trihydroxy-2-methylanthraquinone sublimed readily at 150–170°. The combined sublimates, after one crystallization from chloroform, yielded pure 1:4:5-trihydroxy-2-methylanthraquinone, m.p. 218°, weight 6·1 g. Although this method is somewhat tedious it readily gives a pure product.

The $Na_2CO_3$-extracted chloroform solutions contain, in addition to 1:4:5-trihydroxy-2-methylanthraquinone, other NaOH-soluble colouring matters, which are left in the chloroform mother liquors if method (*a*) is used, or remain unsublimed below 200° if method (*b*) is used. The nature of these colouring matters is being investigated.

The total content of crude mixed colouring matters in the dried mycelium of *P. islandicum*, strain N.R.R.L. 1036, is rather remarkable. In a number of different preparations the average yield obtained was about 20% of the weight of the dried mycelium.

### *General properties of* 1:4:5-*trihydroxy*-2-*methylanthraquinone. Derivatives*

1:4:5-*Trihydroxy*-2-*methylanthraquinone* crystallizes from chloroform or glacial acetic acid in large, dark red, lustrous plates or leaflets, m.p. 218°. (Found: C, 66·8, 66·9; H, 4·0, 3·9; *C*-$CH_3$, 6·0%; $CH_3O$, nil. $C_{15}H_{10}O_5$ requires C, 66·7; H, 3·7; *C*-$CH_3$, 5·6%.) It dissolves fairly readily in chloroform or acetone, is rather less soluble in glacial acetic acid, ethanol and ether, and is almost insoluble in light petroleum. It is insoluble in N-$NaHCO_3$, N-$Na_2CO_3$ and N-$NH_4OH$, but dissolves readily in N-NaOH to a violet solution, the colour of which fades to a pale yellow after a few hours' exposure

to air. Its solution in cold conc. $H_2SO_4$ is bright purple-red in bulk and bluish red in thin layers with a fiery red fluorescence. Its solution in glacial acetic acid is yellow orange in colour with a green fluorescence. It readily sublimes without decomposition in a high vacuum at 160°.

1:4:5-*Triacetoxy*-2-*methylanthraquinone*. A solution of crude 1:4:5-trihydroxy-2-methylanthraquinone (3·36 g.) in 35 ml. of acetic anhydride containing 2% (v/v) conc. $H_2SO_4$ was boiled for 2 min. The cooled brown solution was poured into iced water (1 l.) with stirring. The resulting yellow solid was crystallized twice from ethanol and 4 times from glacial acetic acid to give 1:4:5-*triacetoxy*-2-*methylanthraquinone* as pale yellow needles with a constant m.p. of 208°. (Found: C, 63·7, 63·7; H, 4·2, 4·0%. $C_{21}H_{16}O_8$ requires C, 63·6; H, 4·1%.)

1:4:5-Triacetoxy-2-methylanthraquinone (0·5 g.) was heated with aqueous 2 N-NaOH (30 ml.) on a boiling water bath in an atmosphere of $N_2$. The acetate dissolved in 10 min. to an intensely violet solution. Heating was continued for a further hour when 2 N-HCl (35 ml.) was added. The resulting red amorphous precipitate (0·32 g.) was separated by filtration, washed well with water, dried and crystallized from chloroform. 1:4:5-Trihydroxy-2-methylanthraquinone, weight 0·16 g., m.p. 218°, was thus obtained as dark red plates.

1:4:5-*Trimethoxy*-2-*methylanthraquinone*. 1:4:5-Trihydroxy-2-methylanthraquinone (0·25 g.) was dissolved in boiling anhydrous acetone (25 ml.), and redistilled dimethyl sulphate (1·25 ml.) and anhydrous $K_2CO_3$ (1·25 g.) were added in 0·25 ml. and 0·25 g. portions at suitable intervals during 8 hr. boiling under reflux. The resulting yellow solution was separated by filtration from the mineral salts, which were thoroughly extracted with hot acetone. Evaporation of the solvent *in vacuo* and crystallization of the residue from ethanol gave 1:4:5-*trimethoxy*-2-*methylanthraquinone* (0·20 g.) as orange yellow needles, m.p. 161°, unchanged on further crystallization and sublimation at 150° in a high vacuum. (Found: C, 69·3, 69·2; H, 5·4, 5·4; $CH_3O$, 27·2, 27·7%; mol.wt. (cryoscopic in camphor) 320, 314. $C_{18}H_{16}O_5$ requires C, 69·2; H, 5·2; $3CH_3O$, 29·8%; mol.wt. 312.) In contrast to the parent substance 1:4:5-trimethoxy-2-methylanthraquinone is insoluble in N-NaOH. It dissolves in cold conc. $H_2SO_4$ to a blue solution with a faint purple fluorescence, and in glacial acetic acid to a pale yellow non-fluorescent solution.

### *Oxidation of* 1:4:5-*trihydroxy*-2-*methylanthraquinone. Formation of cynodontin* (*V*)

Finely powdered $MnO_2$ (0·75 g.) was added in portions over a period of 5 min. and with mechanical stirring to a solution of 1:4:5-trihydroxy-2-methylanthraquinone (1 g.) in conc. $H_2SO_4$ (7·5 ml.). The temperature was maintained at 60° and stirring was continued for a further 0·5 hr. The mixture was cooled, poured into water (500 ml.) and then boiled for 10 min. The original brown-violet precipitate became redder on heating. After cooling, the red solid was separated by filtration, washed and dried. The dried material was extracted exhaustively with chloroform in a Soxhlet apparatus, collecting four fractions which were evaporated and crystallized separately giving fraction I, 0·15 g., m.p. 238–240°; II, 0·18 g., m.p. 246–247°; III, 0·23 g., m.p. 247–249°; IV, 0·10 g., m.p. 252–253°; V (from the combined mother liquors), 0·14 g., m.p. 219–224°. Fractions I, II, III and IV were combined and purified by acetylation in the usual way with acetic anhydride and conc. $H_2SO_4$. The resulting acetate was crystallized twice from ethanol yielding 0·40 g. of fine yellow needles, m.p. 228–229°, which did not depress the m.p. of synthetic tetraacetylcynodontin (Anslow & Raistrick, 1940*b*) similarly crystallized. (Found: C, 60·45, 60·8; H, 4·0, 4·0%. Calc. for $C_{23}H_{18}O_{10}$: C, 60·8; H, 4·0%.)

The acetate (0·32 g.) was hydrolyzed with aqueous 2 N-NaOH (20 ml.) on a boiling water bath in an atmosphere of $N_2$. The resulting deep blue solution, on acidification, yielded 0·19 g. of regenerated colouring matter which was crystallized, first from pyridine (20 ml., with charcoal), and then from glacial acetic acid (35 ml.). There was thus obtained 0·11 g. of brownish leaflets with a magnificent bronze lustre which melted at 259·5–260·5°, alone or in admixture with natural cynodontin, m.p. 259·5–260·5°, prepared from *Helminthosporium cynodontis* Marignoni. (Found: C, 63·3, 63·0; H, 3·6, 3·6%. Calc. for $C_{15}H_{10}O_6$: C, 62·9; H, 3·5%.) The two specimens also gave identical reactions in the following tests: N-$Na_2CO_3$, insoluble; N-NaOH, dissolved to a deep blue-violet solution; cold conc. $H_2SO_4$, dissolved to a blue solution with a fine red fluorescence; glacial acetic acid, bluish red solution with a weak greenish yellow fluorescence.

### *Reduction of* 1:4:5-*trihydroxy*-2-*methylanthraquinone. Formation of chrysophanic acid* (*VI*)

A solution of 1:4:5-trihydroxy-2-methylanthraquinone (1 g.) in glacial acetic acid (20 ml.) was boiled for 5 hr. under reflux with red phosphorus (1 g.) and HI (4 ml., sp.gr. 1·7). The mixture was cooled slowly, and the crystals that formed were separated by filtration. When recrystallized from glacial acetic acid (50 ml., with charcoal) there was obtained 0·48 g. of light orange-red plates which had all the colour reactions of chrysophanic acid anthranol and melted at 200–204° (lit. 205–210°).

The anthranol (0·25 g.) was dissolved in hot glacial acetic acid (10 ml.). A solution (1·4 ml.) of $CrO_3$ (10%, w/v) in glacial acetic acid was added with shaking and the mixture was maintained at 60°. A further 1·4 ml. of $CrO_3$ solution were added in portions at intervals, and, after a total oxidation period of 35 min. at 60°, the mixture was cooled. Yellow-orange crystals (0·13 g., m.p. 191–193°) separated, and this material was purified by sublimation in a high vacuum at 140–160°. The orange-yellow sublimate was dissolved in chloroform, extracted three times with 10 ml. of 0·5 N-aqueous $Na_2CO_3$, and then washed with water. After removal of the chloroform, the resulting solid was crystallized from ethanol, yielding glistening orange-yellow plates (0·06 g., m.p. 194°). (Found: C, 70·6, 70·8; H, 4·2, 4·3%. Calc. for $C_{15}H_{10}O_4$: C, 70·9; H, 4·0%.) The substance proved to be chrysophanic acid, a pure specimen of which was prepared from a sample of Kahlbaum's chrysophanic acid kindly given to us by Dr H. D. Springall, Department of Chemistry, University of Manchester. This authentic specimen melted at 195° and a mixture of the two specimens melted at 195°. The two specimens also gave identical reactions in the following tests: N-$Na_2CO_3$, insoluble; N-NaOH, dissolved to a red solution, changing slowly to orange red with the formation of a red precipitate; cold conc. $H_2SO_4$, dissolved to a red non-fluorescent solution; glacial acetic acid, dissolved to a yellow solution.

The reduction product, chrysophanic acid (0·13 g.), was acetylated in the usual way with acetic anhydride (2 ml.) containing 1 drop of conc. $H_2SO_4$. The solid that separated on adding ice was crystallized from ethanol (with charcoal) and gave yellow leaflets, m.p. 207–208°. (Found: C, 67·1; H, 4·3%. Calc. for $C_{19}H_{14}O_6$: C, 67·45; H, 4·2%.) It was compared with an authentic specimen of diacetyl chrysophanic acid, kindly supplied by the Wellcome Research Institution and prepared originally by Dr F. B. Power. This specimen melted at 208–209°, and a mixture of the two at 208–209°.

### *Comparisons of* 1:4:5-*trihydroxy*-2-*methylanthraquinone*

(*a*) *With nataloemodin.* A specimen of methylnataloemodin (nataloemodin monomethyl ether) prepared originally by Léger (1902, 1905) was kindly given to us by Dr T. E. Wallis, Curator of the Museum of the Pharmaceutical Society, London. A solution of methylnataloemodin (54 mg.) in 5 ml. of equal volumes of glacial acetic acid and constant b.p. aqueous HBr (46–48%) was boiled under reflux for 6 hr. The initially yellow solution slowly became brown and on standing overnight deposited nataloemodin in orange-brown needles in almost theoretical yield. These were recrystallized from acetic acid (60% v/v, 16 ml.) and yielded pure nataloemodin (0·04 g.) as fine golden yellow needles, m.p. 216°. A mixture of nataloemodin with 1:4:5-trihydroxy-2-methylanthraquinone, m.p. 218°, melted at 185–192°. Nataloemodin gave the following colour reactions: N-$Na_2CO_3$, readily dissolves to a cerise solution; 0·5N-NaOH, violet solution not fading quickly; cold conc. $H_2SO_4$, red-violet solution almost indistinguishable from aqueous $KMnO_4$; glacial acetic acid, yellow non-fluorescent solution. The two substances are clearly quite different compounds.

(*b*) *With helminthosporin* (*III*). A mixture of 1:4:5-trihydroxy-2-methylanthraquinone, m.p. 218°, with helminthosporin, m.p. 225–226°, from *Helminthosporium gramineum* Rabenhorst melted at 192–200°; a mixture of 1:4:5-triacetoxy-2-methylanthraquinone, m.p. 208°, with triacetyl helminthosporin, m.p. 223–225°, melted at 199–203°. It is thus clear that the two substances are quite different compounds, although in comparative colour tests with N-NaOH, cold conc. $H_2SO_4$ and glacial acetic acid the colours given by the two substances were almost indistinguishable.

## SUMMARY

1. The dried mycelium of laboratory cultures of *Penicillium islandicum* Sopp has been shown to contain about 20% of its weight of a complex mixture of colouring matters.

2. One constituent of this mixture has been isolated in a pure form from five different strains of *P. islandicum* and has been shown to be the hitherto undescribed 1:4:5-*trihydroxy*-2-*methyl*-*anthraquinone*.

3. 1:4:5-Trihydroxy-2-methylanthraquinone crystallizes in lustrous dark red plates, m.p. 218°, and forms a *triacetate*, pale yellow needles, m.p. 208°, and a *trimethyl ether*, orange yellow needles, m.p. 161°.

4. On oxidation with manganese dioxide and concentrated sulphuric acid, 1:4:5-trihydroxy-2-methylanthraquinone yields cynodontin; on reduction with concentrated hydriodic acid and red phosphorus and oxidation of the resulting anthranol with chromic acid, chrysophanic acid is formed.

5. The close structural relationship existing between a number of mould colouring matters is discussed.

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# Studies in Detoxication

## 21. THE FATES OF QUINOL AND RESORCINOL IN THE RABBIT IN RELATION TO THE METABOLISM OF BENZENE

By G. A. GARTON and R. T. WILLIAMS
*Department of Biochemistry, University of Liverpool*

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The study of the metabolic fates of quinol and resorcinol was undertaken to elucidate certain aspects of the metabolism of benzene and phenol (see Porteous & Williams, 1949*a*, *b*). We have already reported on the fate of catechol (Garton & Williams, 1948), and a further study of the fate of phenol is in progress. Quinol is also of importance in industry as a photographic chemical and as a food preservative. Its position as an industrial hazard has been discussed recently by Sterner, Oglesby & Anderson (1947).

There is little exact information concerning the fate of these two phenols in the body. Baumann & Preusse (1879) found that a dog given 0·5 g. quinol excreted a quinol ethereal sulphate though no free quinol could be detected. von Mering (1876) also demonstrated the presence of a quinol ethereal sulphate in the urine of rabbits fed with arbutin (quinol monoglucoside). Külz (1890) found that quinol-fed rabbits excreted a glucuronide. Baumann (1878–9) reported that resorcinol raised the ethereal sulphate output in dogs, and Külz (1890) showed that it formed a glucuronide in rabbits and hares.

## EXPERIMENTAL

### *Quantitative experiments*

The rabbits (giant chinchilla) used were kept on a diet of Lever's cubes (50 g./day) and water *ad lib.* Quinol (m.p. 169°) and resorcinol (m.p. 110°), dissolved in water, were fed by stomach tube. Doses up to 0·4 g./kg. of quinol and 0·5 g./kg. of resorcinol could be administered without apparent toxic effect. Doses of 0·45 g./kg. quinol and 0·6 g./kg. resorcinol caused temporary muscular twitching and an increased rate of respiration. In most of the present experiments doses of 0·1–0·2 g./kg. were used.

*Glucuronic acid* and *ethereal sulphate* excretions were determined as described in earlier papers in this series (Hanson, Mills & Williams, 1944; Williams, 1938). The results, given in Table 1, show that with quinol 30% of the dose is excreted as an ethereal sulphate and 43% as a monoglucuronide, and with resorcinol 13·5% is excreted as a sulphate and 52% as a monoglucuronide.

Table 1. *The excretion of glucuronic acid and ethereal sulphate by rabbits receiving quinol or resorcinol orally*

(The dose of dihydroxybenzene was 100 mg./kg. of body wt.)

| | | | Average normal values | | 'Extra' excretion | | Percentage of dose excreted | | |
|---|---|---|---|---|---|---|---|---|---|
| Rabbit no. | Wt. (kg.) | Dose (mg.) | Ethereal sulphate (mg. $SO_3$/day) | Glucuronic acid (mg./day) | Ethereal sulphate (mg. $SO_3$/day) | Glucuronic acid (mg./day) | As mono-sulphate | As mono-glucuronide | Total conjugation |
| Quinol fed: | | | | | | | | | |
| 122 | 3·15 | 630* | 25·1 | — | 129·4 | — | 28·3 | — | — |
| 123 | 3·50 | 700* | 14·7 | — | 200·3 | — | 39·3 | — | — |
| 124 | 3·05 | 710† | 13·9 | — | 126·8 | — | 24·6 | — | — |
| 98 | 3·15 | 315 | 17·1 | 168 | 86·7 | 232 | 37·8 | 41·8 | 79·6 |
| 90 | 2·95 | 295 | — | 116 | — | 184 | — | 35·4 | — |
| 81 | 2·85 | 285 | — | 150 | — | 213 | — | 42·4 | — |
| 124 | 2·90 | 290 | 16·7 | 126 | 56·2 | 250 | 26·6 | 48·9 | 75·5 |
| 125 | 3·10 | 310 | 18·3 | 155 | 56·2 | 231 | 24·9 | 42·2 | 67·1 |
| 126 | 3·05 | 305 | 19·2 | 156 | 62·6 | 245 | 28·3 | 46·6 | 74·8 |
| Resorcinol fed: | | | | | | | | | |
| 124 | 2·85 | 285 | 13·8 | 106 | 27·8 | 272 | 13·4 | 54·1 | 67·5 |
| 125 | 3·25 | 325 | 15·8 | 105 | 31·2 | 295 | 13·2 | 51·5 | 64·7 |
| 126 | 3·10 | 310 | 15·2 | 107 | 39·7 | 277 | 14·0 | 50·6 | 64·6 |

* Dose 200 mg./kg. † Dose 230 mg./kg.

### *Isolation of metabolites*

*Metabolites of quinol*

*Quinol urine* is normal in colour when freshly voided, but darkens considerably on standing. The slightly alkaline urine gives a dark green-brown colour with 2% $FeCl_3$. It reduces very slightly to Fehling's and Benedict's reagents and gives an intense Tollens test for glucuronic acid.

*Isolation of free quinol.* The 24 hr. urine (340 ml.), collected after feeding a total of 4 g. quinol to three rabbits, was filtered, treated with 0·1 g. $Na_2S_2O_4$ and extracted continuously for 6 hr. with peroxide-free ether. The extract was evaporated at 30° to a clear pale-yellow gum (90 mg.). The gum was dissolved in 5 ml. 2·5N-NaOH and treated with 0·2 g. *p*-toluenesulphonyl chloride in 5 ml. acetone. After it had been shaken for 10 min. the mixture was poured into 50 ml. water. The crystals (10 mg., 0·065% of dose) which formed were collected and dried and identified as quinol di-*p*-toluenesulphonate, m.p. 155° alone and 157° mixed with an authentic specimen, m.p. 159°. A separate experiment showed that quinol added to normal urine could be recovered quantitatively by ether extraction after the addition of $Na_2S_2O_4$.

*The glucuronide of quinol. Isolation of the quinol glucuronide gum.* A 24 hr. urine (945 ml.) was collected after feeding a total of 12 g. quinol to twelve rabbits and the basic lead acetate precipitate prepared in the usual manner. The precipitate was well washed with water and Pb removed with $H_2S$. The Pb-free filtrate was aerated, treated with charcoal, filtered and evaporated to a gum (18 g.) *in vacuo* at 50–60°. The gum was dissolved in 10 ml. absolute ethanol and kept at 0° overnight. A precipitate (0·5 g.) of inorganic material was removed, and the solution was then evaporated to dryness. The gum, presumably *quinol glucuronide*, was purified by dissolving in ethanol, filtering and evaporating several times and yielded 16·5 g. of a clear brown gum. It was acid to litmus, gave a pale green colour with $FeCl_3$ which deepened on making slightly alkaline with $NaHCO_3$ and an intense Tollens naphthoresorcinol test. It was insoluble in ether, benzene and light petroleum.

*Quinol monoglucuronide methyl ester.* The gum (3 g.) was dissolved in 50 ml. ethanol and 200 ml. of a saturated solution of diazomethane in ether added. The mixture was kept overnight, filtered from a little sludge and evaporated to dryness. The treatment with diazomethane was repeated twice. Finally a clear yellow, neutral, ether-soluble gum (2·47 g.) was obtained which could not be induced to crystallize. The p-*hydroxyphenylglucuronide methyl ester* gave a blue colour with $FeCl_3$, indicating the presence of a free phenolic OH group. (Found: $OCH_3$, 10·2. $C_{13}H_{16}O_8$ requires $OCH_3$, 10·3%.)

*Acetylation of quinol glucuronide methyl ester.* The above methyl ester (2·47 g.) was dissolved in 10 ml. pyridine and 15 ml. acetic anhydride. After it had been kept overnight at room temperature the mixture was poured into 100 ml. of ice water. After the mixture had been stirred for some time at 0° crystallization occurred. The crystals (3·25 g.; m.p. 148°) were collected and recrystallized from ethanol. The p-*acetoxyphenyl-triacetyl*-D-*glucuronide methyl ester* formed long, colourless needles, m.p. 151°, $[\alpha]_D^{19°} - 21·8°$ (*c*, 1 in acetone). (Found: C, 53·7; H, 5·1; $OCH_3$, 6·9. $C_{21}H_{24}O_{12}$ requires C, 53·85; H, 5·2; $OCH_3$, 6·6%.) The compound was insoluble in water, sparingly soluble in ethanol, soluble in ether and readily soluble in acetone. It gave the naphthoresorcinol reaction on prolonged heating (5 min.) with the reagents.

*Hydrolysis of acetylated quinol monoglucuronide methyl ester.* *p*-Acetoxyphenyl-triacetylglucuronide methyl ester (1·5 g.) in 15 ml. ethanol and 100 ml. 4N-HCl was heated under reflux for 4 hr. on the water bath. After cooling, the brown solution was extracted with 5 × 50 ml. peroxide-free ether. The extracts were evaporated to dryness *in vacuo* and the partially crystalline residue dissolved in 10 ml. 2·5N-NaOH and treated with 0·5 g. *p*-toluenesulphonyl chloride. After shaking for 20 min. the mixture was poured into 150 ml. cold water. The crystalline precipitate (315 mg.) of quinol di-*p*-toluenesulphonate was recrystallized from absolute ethanol to give colourless prisms, m.p. and mixed m.p. 159°.

*The ethereal sulphate fraction of quinol urine.* If it is assumed that quinol forms a monosulphate, then, according to Table 1, the proportion of quinol conjugated with sulphuric acid is twice that of catechol (Garton & Williams, 1948) or resorcinol. It was, therefore, necessary to show whether quinol monosulphuric acid or quinol disulphuric acid is excreted by rabbits.

Attempts to isolate the ethereal sulphate and compare it with synthetic material (see below) were unsuccessful, but we proved it to be a monosulphate by the following procedure. The urine (1025 ml.), collected after feeding a total of 10 g. quinol to ten rabbits, was treated with 0·5 g. $K_2CO_3$ and reduced *in vacuo* at 40° to 250 ml. The resulting dark brown liquid was clarified with 0·25 g. $Na_2S_2O_4$ and saturated with $(NH_4)_2SO_4$; 250 ml. acetone were added and the mixture filtered at the pump, the precipitated $(NH_4)_2SO_4$ being washed with 150 ml. acetone. The combined filtrates were transferred to a separating funnel and the aqueous layer removed. The acetone solution was treated with 0·5 g. $K_2CO_3$ and concentrated *in vacuo* at 40° to 200 ml. This was now poured into 2 l. dry acetone. A dark brown viscous layer containing glucuronides separated and was discarded. The clear golden acetone layer was again reduced *in vacuo* at 40° to 150 ml. Glucuronides were still present and so the solution was poured into 1 l. dry acetone and the brown gum of glucuronide again removed. The acetone solution was again evaporated at 40° to a gummy crystalline mass. The product was practically free from glucuronides, and contained no inorganic sulphate. The crystals in it were identified as urea. This material contained the ethereal sulphates of quinol urine, but these could not be isolated. It was, therefore, dissolved in 100 ml. 50% aqueous ethanol, and its content of free and total quinol and ethereal sulphate estimated as follows.

Quinol was estimated by the iodometric method of Wieland (1910), as described by Neuberger (1947) for the determination of homogentisic acid in urine. Urea does not interfere with the determination. The above ethereal sulphate fraction contained 55 mg. of free quinol. For the estimation of total quinol, 5 ml. of the ethereal sulphate fraction were boiled under reflux with 10 ml. of 0·3N-HCl for 30 min., cooled and the quinol estimated iodometrically. The combined quinol found was 1·045 g.

A gravimetric determination of ethereal sulphate showed that the fraction contained 0·8561 g. $SO_3$. Thus the ratio ethereal sulphate $SO_3$/combined quinol in the fraction is 0·8561/1·045 = 0·82. For quinol monosulphuric acid the calculated ratio is 0·73, whereas for quinol disulphuric acid

it is 1·45. These results indicate that the ethereal sulphate of quinol urine is quinol monosulphuric acid.

*Search for quinol oxidation products.* In an earlier paper (Garton & Williams, 1948) we showed that, in the rabbit, hydroxyquinol is an oxidation product of catechol. Since catechol, quinol and hydroxyquinol are metabolites of benzene (Porteous & Williams, 1949*b*), it is important to know whether quinol is also oxidized to hydroxyquinol. A careful search was made for this phenol in the urine collected 1, 2 and 3 days after feeding a total of 4 g. quinol to three rabbits. No trace of free or combined hydroxyquinol was found. The colour reactions used have been described in earlier papers (Garton & Williams, 1948; Porteous & Williams, 1949*b*).

*Metabolites of resorcinol*

*Resorcinol urine* is slightly darker than normal rabbit urine, but does not darken on standing as does quinol urine. The slightly alkaline urine is non-reducing, gives no colour with $FeCl_3$, but gives a strong naphthoresorcinol reaction. It contains free resorcinol, giving a purplish red colour in the 2:6-dichloroquinone chloroimide test of Porteous & Williams (1949*b*).

*Isolation of free resorcinol.* Resorcinol (1 g.) was fed to each of six rabbits. The slightly alkaline 24 hr. urine (625 ml.) was filtered through glass wool and then extracted continuously for 8 hr. with peroxide-free ether. Evaporation of the extract at 20° yielded a partially crystalline product (1·97 g.) from which 0·5 g. resorcinol (m.p. and mixed m.p. 110°, after crystallization from dry benzene) was obtained. The mother liquors, on benzoylation, yielded 0·255 g. of resorcinol dibenzoate (m.p. and mixed m.p. 117°). A further 8 hr. extraction removed all the free resorcinol from the urine and 0·27 g. of the dibenzoate was obtained. Thus a total of 11·4% of the resorcinol fed was recovered from the urine in the free state.

*The glucuronide of resorcinol: isolation of the resorcinol glucuronide gum.* The glucuronide gum (22 g.) was prepared by the usual basic lead acetate procedure from the 24 hr. urine (1·5 l.) of eight rabbits which had collectively received 12 g. of resorcinol. The gum was purified by dissolution in absolute ethanol and filtering. In this way 23·5 g. of a viscous brown gum were obtained consisting mainly of *resorcinol monoglucuronide*. This gum was acidic, non-reducing, gave an olive-green colour with $FeCl_3$ and an intense naphthoresorcinol reaction. It was readily soluble in water, ethanol and ethyl acetate. It could not be crystallized, or induced to give crystalline salts with organic bases.

Since resorcinol monoglucuronide is a phenol in which the position para to the phenolic OH group is unsubstituted, it should give a coloration with 2:6-dichloroquinone chloroimide. In fact the gum gives an intense purple colour with this reagent in slightly alkaline solution ($NaHCO_3$) and at all values of pH up to 10. This reaction in itself shows that the glucuronide of resorcinol is a monoglucuronide. Resorcinol itself gives a purplish red colour with 2:6-dichloroquinone chloroimide. The quinol monoglucuronide gum which has already been described gives no colour with 2:6-dichloroquinone chloroimide, a result to be expected since quinol glucuronide is a *p*-substituted phenol.

*Resorcinol monoglucuronide methyl ester.* The above gum (5 g.) was methylated in ethanol with ethereal diazomethane. m-*Hydroxyphenylglucuronide methyl ester* was obtained as an ether-soluble, neutral, clear brown gum (Found: $OCH_3$, 11·4. $C_{13}H_{16}O_8$ requires $OCH_3$, 10·3%), which gave a transient purple colour with $FeCl_3$.

*Acetylation of resorcinol glucuronide methyl ester.* The ester (3·6 g.) was acetylated at room temperature with 10 ml. pyridine and 15 ml. acetic anhydride. On pouring the mixture into water a yellow oil separated. The oil was taken up in 50 ml. $CHCl_3$ and the solution washed successively with 10% $Na_2CO_3$, 2N-HCl and water. After drying over anhydrous $CaCl_2$, the chloroform was evaporated, leaving 4 g. of a pale yellow gum which did not crystallize. The acetylation and extraction procedure was therefore repeated, but again a yellow gum was obtained. The gum was dissolved in 30 ml. ethanol and water was added dropwise with stirring. After vigorous scratching the precipitated material crystallized and eventually 3·16 g. of small colourless needles, m.p. 112°, were obtained. The m-*acetoxyphenyl-triacetyl-*D*-glucuronide methyl ester* was recrystallized from ethanol, forming small needles, m.p. 113–114°, $[\alpha]_D^{20°}$ −24·5° (*c*, 2 in acetone). (Found: C, 54·25; H, 5·2; $OCH_3$, 6·9; $CH_3CO$, 39·8. $C_{21}H_{24}O_{12}$ requires C, 53·85; H, 5·2; $OCH_3$, 6·6, $CH_3CO$, 36·8%.) The compound was soluble in ethanol, acetone and chloroform, but insoluble in water. It gave a strong naphthoresorcinol reaction when boiled for 2–3 min. with the reagents.

*Hydrolysis of the acetylated resorcinol monoglucuronide methyl ester.* The ester (3 g.) was refluxed on a water bath with 15 ml. ethanol and 100 ml. 4N-HCl for 2·5 hr. A red-brown solution was formed which, on cooling, deposited a reddish flocculent precipitate. This precipitate is presumably the result of the condensation of the free resorcinol with the free glucuronic acid formed during hydrolysis of the ester (cf. the naphthoresorcinol reaction). The solution was extracted with 4 × 75 ml. ether and the combined extracts dried over anhydrous $Na_2SO_4$. Removal of the ether at 30° left a pale brown gum (1·53 g.). On benzoylating the gum and pouring the product into water, an oil separated. This was dissolved in 30 ml. acetone which was then poured into 100 ml. water giving 350 mg. of small colourless plates of resorcinol dibenzoate, which, after recrystallization from aqueous ethanol, had m.p. and mixed m.p. 117° with authentic material.

The residual solution, after removal of resorcinol by ether, contained glucuronic acid as indicated by the Tollens test and by the reduction of Benedict's and Fehling's solutions.

*Search for resorcinol oxidation products.* Resorcinol urine was also carefully examined for hydroxyquinol, pyrogallol and phloroglucinol. No trace of any of these phenols was found in either the ethereal sulphate or glucuronide fraction of the urine collected 24 or 48 hr. after feeding a total of 6 g. resorcinol to six rabbits.

The colour tests used were those described by Porteous & Williams (1949*b*), together with a specific colour reaction for phloroglucinol. In this test traces of phloroglucinol give a red-orange colour with 0·25% aqueous quinol and 0·5N-KOH (cf. Porteous & Williams, 1949*a*).

## DISCUSSION

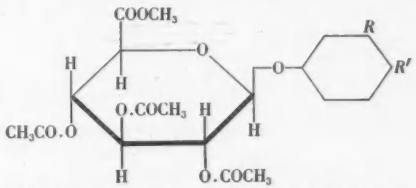
The quantitative aspects of the metabolic fates of the *o*-, *m*- and *p*-dihydroxybenzenes are summarized in Table 2, the values for catechol being quoted from Garton & Williams (1948). Quinol and catechol

Table 2. *The metabolism of the isomeric dihydroxybenzenes in the rabbit*

| Compound | Conjugation (% of dose): As ethereal sulphate (*E*) | As glucuronide (*G*) | Total | *G*/*E* | Other metabolites detected |
|---|---|---|---|---|---|
| Catechol | 18 | 70 | 88 | 3·9 | Free catechol (2% isolated), traces of hydroxyquinol as an ethereal sulphate |
| Resorcinol | 13·5 | 52 | 65·5 | 3·9 | Free resorcinol (11·4% isolated) (combined + free = 77%) |
| Quinol | 30 | 43 | 73 | 1·4 | Free quinol in traces (0·065% isolated) |

are excreted almost entirely conjugated and although resorcinol is also highly conjugated, appreciable amounts (11–12% by isolation) are excreted in the free state. The ratio glucuronide/ethereal sulphate is approximately 4 for both catechol and resorcinol, but only 1·4 for quinol, which is more highly conjugated with sulphate than its isomers. The proportion of the sulphate conjugation of quinol is twice that of its isomers and at first we suspected that quinol was being excreted as a disulphuric ester. The evidence presented, however, indicates that the ethereal sulphate is quinol monosulphuric acid. A consideration of the figures for the sulphate conjugation of resorcinol suggests that there is no reason to believe that resorcinol forms other than a monosulphuric ester.

The glucuronides of resorcinol and quinol have been shown to be monoconjugates by the isolation and characterization of the crystalline acetylated methyl esters (I) and (II) (*m*- and *p*-acetoxyphenyl-2:3:4-triacetyl-β-D-glucuronide methyl esters).




I ($R$=$CH_3COO$; $R'$=H) II ($R$=H; $R'$=$CH_3COO$)

Our proof of the structure of catechol monoglucuronide (Garton & Williams, 1948) depended on the fact that, on methylation with ethereal diazomethane, the glucuronide was converted to *o*-methoxyphenylglucuronide methyl ester. Hydrolysis of the crystalline triacetyl derivative of this ester yielded catechol monomethyl ether, thus proving that the original glucuronide contained one free phenolic hydroxyl group. In the present work, however, we found that when quinol and resorcinol glucuronides were methylated with diazomethane only the carboxyl groups were methylated. The non-crystalline methyl esters of quinol and resorcinol glucuronides obtained gave colours with ferric chloride, thus showing the presence of a free phenolic hydroxyl group. The proof that these glucuronides were monoconjugates, therefore, depends on the elementary analysis of the crystalline acetylated methyl esters (I) and (II) and on colour tests for free phenolic hydroxyl groups. The purified non-crystalline quinol monoglucuronide gave a pale green colour with ferric chloride, its methyl ester a blue colour, whereas non-crystalline resorcinol monoglucuronide gave an olive-green and its methyl ester a transient purple colour. Further evidence that resorcinol glucuronide contains a free phenolic group was obtained by the use of 2:6-dichloroquinone chloroimide (see p. 236).

Neither quinol nor resorcinol undergoes further oxidation *in vivo* as does catechol. We searched very carefully for trihydric phenols, but we found no indication of their presence. In an earlier paper (Porteous & Williams, 1949*b*) it was shown that phenol, catechol, quinol and hydroxyquinol were oxidation products of benzene in the rabbit. The present paper shows that hydroxyquinol is not a metabolite of quinol, though we have shown it to be formed from catechol (Garton & Williams, 1948). Thus the hydroxyquinol in benzene urine must be derived from catechol. A detailed study of the metabolism of phenol in the rabbit (Garton & Williams, unpublished) has shown that phenol gives rise to catechol, quinol and hydroxyquinol. Porteous & Williams (1949*b*) suggested that benzene was oxidized in the rabbit as follows:

benzene → phenol → catechol → hydroxyquinol.
↓
quinol

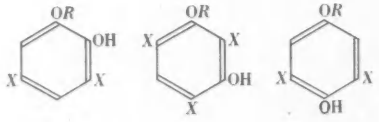
This scheme has now substantial experimental support.

The present work also raises the question, why is catechol oxidized to hydroxyquinol, whereas quinol and resorcinol are not? There are three possible answers to this question.

First, the enzymic systems which occur in animal tissues may oxidize only catechol. It is known, for example, that the tyrosinase system will oxidize catechol, but not quinol and resorcinol (Nelson & Dawson, 1944; Sumner & Somers, 1947). Nevertheless, Cadden & Dill (1942) have obtained from pig kidney a polyphenolase which oxidizes both

catechol and quinol. This enzyme resembles the plant enzyme laccase which oxidizes quinol and catechol to quinones, but not resorcinol (Yakushiji, 1940). Samisch (1937) claims that lemon leaves contain a metaphenolase which oxidizes resorcinol very slowly.

A second possible explanation may be derived from a consideration of the structures of the three phenols and their monoconjugates. It is well known that phenols are oxidized either *o*- or *p*- to the existing —OH group. In the formulae (III), (IV) and (V), the *o*- and *p*-positions are marked with *X*




(III) (IV) (V)

and it is to be noted that in the resorcinol (IV) and quinol (V) derivatives, the positions *X* are all ortho to an existing group. There may, therefore, be steric hindrance to oxidation in these positions. There is one position *X* in the catechol derivative (III) which is not subjected to steric hindrance by ortho substituents, and catechol is the only dihydric phenol which is metabolized to hydroxyquinol. More information is required, however, on the metabolism of hydroxyquinol before the plausibility of this explanation can be assessed. Preliminary work on hydroxyquinol is in hand.

A third explanation takes into account the time factor. If quinol and resorcinol were excreted more rapidly than catechol, then it is possible that catechol, because of its longer stay in the body, would have more chance of being oxidized than the others. We have no exact data on relative rates of excretion of these phenols, except that we observed that in the doses used in the present work all three were almost completely excreted within 24 hr. of dosing.

## SUMMARY

1. The metabolism of quinol and resorcinol in the rabbit has been studied.

2. Quinol is excreted almost entirely as monoglucuronide (43 %) and monosulphate (30 %). Only traces of free quinol are excreted.

3. Resorcinol is excreted as monoglucuronide (52 %), monosulphate (13·5 %) and in the free state (11·4 %).

4. Quinol and resorcinol monoglucuronides were isolated and characterized as the crystalline methyl esters of the acetoxyphenyltriacetyl glucuronides.

5. Neither quinol nor resorcinol is oxidized *in vivo* to trihydric phenols or other substances.

6. The results have been discussed in relation to the metabolism of benzene, phenol and catechol.

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# Studies in Detoxication

## 22. THE METABOLISM OF PHENACETIN (*p*-ETHOXYACETANILIDE) IN THE RABBIT AND A FURTHER OBSERVATION ON ACETANILIDE METABOLISM

By J. N. SMITH and R. T. WILLIAMS
*Department of Biochemistry, University of Liverpool*

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We have shown that acetanilide is converted in the rabbit mainly into the glucuronide and ethereal sulphate of *p*-acetamidophenol (Smith & Williams, 1948), not more than 6–7% being excreted as compounds containing free aromatic amino groups. Phenacetin has antipyretic and analgesic properties similar to those of acetanilide, but is less toxic. The similar pharmacological properties suggest that phenacetin may give rise to the same metabolites as acetanilide, and in this paper we shall show that this suggestion is true.

Earlier workers showed that phenacetin caused an increased output of ethereal sulphate in man, the dog and the rabbit (Müller, 1888; Mörner, 1889; Baccarani, 1900; Hinsberg & Kast, 1887; Mahnert, 1888) and Mörner isolated potassium *p*-acetamidophenylsulphate from the urine of patients receiving phenacetin orally. The presence of a glucuronide and of compounds of *p*-aminophenol in phenacetin urine from man, the dog and the rabbit was also suggested by this early work. According to Müller (1888) no unchanged phenacetin is excreted, but Mörner (1889) found small amounts of phenetidine in human urine. Hinsberg & Kast (1887) observed that a dog which had received 3 g. of phenacetin orally excreted urine which strongly reduced alkaline copper solutions; the significance of this observation will be discussed later.

## EXPERIMENTAL

*The quantitative analyses* carried out on phenacetin urine are quoted in the succeeding paper (Smith & Williams, 1949*a*), in which analytical figures for a number of related aromatic amines are discussed together. At this point it may be mentioned that phenacetin undergoes only a very slight deacetylation in the rabbit and it is to be expected, therefore, that the major metabolites will be aromatic acetamido compounds.

*The nature of phenacetin urine.* The urine of rabbits receiving 0·3–0·7 g./kg. phenacetin orally had pH *c.* 8 and was normal in appearance. It did not reduce Benedict's reagent immediately, but a slight reduction was apparent on allowing the test mixture to stand. It gave no colour with $FeCl_3$ and only a very slight red colour in the diazo test (diazotization and coupling with 1-naphthyldimethylamine). The urine yielded no ether-soluble material on shaking with ether in a separating funnel.

### 1. *The ethereal sulphate fraction of phenacetin urine*

(*a*) *Detection of free* p-*phenetidine*

A 24 hr. urine (1 l.), collected after the feeding of 15 g. phenacetin, was evaporated *in vacuo* at 40–50° to 200 ml. Phenetidine was detected in the distillate by the permanganate-like colour it gives with $FeCl_3$, but it could not be isolated in sufficient amount for identification. The concentrated urine was saturated with $(NH_4)_2SO_4$ and then extracted with 2 × 200 ml. portions of acetone. The acetone extract was separated (leaving an aqueous layer *G* containing glucuronide, see section 2 (*b*) (i)), neutralized with solid $K_2CO_3$, filtered and reduced to 50 ml. *in vacuo*. The residue *B* was shaken with 500 ml. dry acetone and the supernatant layer separated and reduced to 50 ml. (*C*) *in vacuo*. *C* was now extracted with ether and the extract on evaporation yielded a small oily residue (*D*) which appeared by colour tests to be phenetidine. *D* was dissolved in 1 ml. 2N-HCl, filtered, neutralized with dilute $Na_2CO_3$ solution and extracted with ether. The extract was evaporated, and the residue treated with a little acetic anhydride and dilute $Na_2CO_3$ solution. Phenacetin separated and, recrystallized from a little hot water, had m.p. and mixed m.p. 132° (yield, 10 mg., or 0·07% of dose).

(*b*) *Isolation of* p-*aminophenol*

The ether-extracted concentrate *C* (above) was a syrupy liquid. It was practically free of glucuronides and gave no precipitate with $BaCl_2$ until boiled with dilute HCl. It did not reduce ammoniacal $AgNO_3$ and gave no colour in the diazo test, but both these tests became positive after hydrolysis. This fraction, therefore, contained the ethereal sulphate of an acetylated aminophenol.

A 30 ml. portion of *C* was boiled for 10 min. with 3 ml. of conc. HCl. The mixture was cooled and extracted with ether. The extract was evaporated to 5 ml. and stirred with dilute $Na_2CO_3$ solution and a little acetic anhydride. The solid which separated was recrystallized from benzene and the crystals (plates) obtained were identified as *ON*-diacetyl-*p*-aminophenol, m.p. and mixed m.p. 150°. (Found: N, 7·3. Calc. for $C_{10}H_{11}O_3N$: N, 7·25%.) The yield was 120 mg., or 1·3% of dose. The ethereal sulphate fraction, therefore, contained *p*-acetamidophenylsulphuric acid.

### 2. *The glucuronide fraction of phenacetin urine*

(*a*) *Isolation of* p-*acetamidophenylglucuronide*

The 24 hr. urine (400 ml.) from two rabbits, which had each received 2 g. (0·7 g./kg.) of phenacetin, was acidified

with a little glacial acetic acid and treated with 100 ml. saturated lead acetate solution. The precipitate was discarded. The filtrate was made just alkaline with ammonia (sp.gr. 0·88) and the precipitate which formed was collected, washed well with water, then suspended in water and treated with a current of $H_2S$ to remove Pb. After filtering from PbS, the filtrate was reduced *in vacuo* at 45° to 5 ml. Addition of ethanol threw down a ropy precipitate which dissolved on warming and came down again on cooling as a gum containing some crystals. The whole was, therefore, reduced *in vacuo* to 5 ml. and then mixed with 4 ml. of benzylamine. This mixture was now diluted to 1 l. by alternate additions of ethyl acetate and ethanol so that the last addition of ethyl acetate produced a slight cloudiness. After keeping at 0° for 3 hr. the crystalline precipitate (1·65 g.) was filtered off and the mother liquor diluted to 2 l. with ethyl acetate. On keeping overnight at 0° a further 0·65 g. of crystals was obtained. Concentration and redilution of the mother liquor yielded a further 0·6 g. These crystals were identified as the benzylamine salt of *p*-acetamidophenylglucuronide, m.p. and mixed m.p. 195–197° after recrystallization from aqueous ethanol, $[\alpha]_D^{15°} - 60°$ (*c*, 1·4 in water). (Found: N, 6·2. Calc. for $C_{21}H_{26}O_8N_2.H_2O$: N, 6·2%.) (See Smith & Williams, 1948.) The yield corresponded to 32% of the dose of phenacetin; quantitative estimations showed that 47% of phenacetin is excreted as a glucuronide (Smith & Williams, 1949*a*).

(*b*) *The detection of a labile glucuronide*

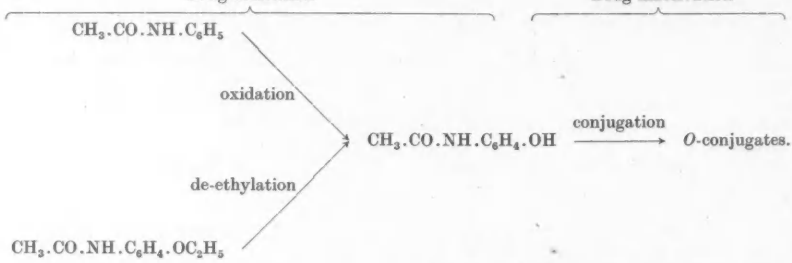
In the succeeding papers (Smith & Williams, 1949*a*, *b*) it is suggested that the main metabolites of aniline and phenetidine in the rabbit are labile glucuronides. Now both acetanilide (Smith & Williams, 1948) and phenacetin undergo a very slight deacetylation in the rabbit and should, therefore, give rise to small amounts of these labile glucuronides. These substances can be detected in urine because they yield, in the presence of *p*-toluidine and $NH_4^+$ ions, a crystalline complex of *p*-toluidine and ammonium glucuronate. The nature of this complex is discussed by Smith & Williams (1949*b*).

(i) *Detection of a labile glucuronide in phenacetin urine.* The aqueous residue *G* (see Section 1 (*a*)) left after removal of the sulphate fraction from phenacetin urine by acetone consisted of 50 ml. of urine saturated with $(NH_4)_2SO_4$. Into this was stirred 1 g. of *p*-toluidine dissolved in a little acetone. After several hours the crystalline precipitate which had separated was filtered off and washed with acetone to remove excess *p*-toluidine. The crystals (50 mg.) were recrystallized from water and had m.p. 125–128°, not depressed on admixture with the *p*-toluidine-ammonium glucuronate complex from aniline or phenetidine urine. The compound rapidly reduced Benedict's reagent and gave the Tollens naphthoresorcinol reaction very readily.

(ii) *From acetanilide urine.* A 24 hr. urine (800 ml.) from rabbits which had collectively received 10 g. acetanilide was reduced *in vacuo* at 40–50° to 160 ml. The concentrate was acidified with a little 2N-HCl, saturated with $(NH_4)_2SO_4$ and extracted with acetone (2 × 150 ml.). The aqueous layer was separated and treated with an ethanolic solution of 5 g. *p*-toluidine followed by 50 ml. ethanol. On keeping this mixture, solid $(NH_4)_2SO_4$ separated and settled on the bottom of the vessel whereas crystals of the *p-toluidine-ammonium glucuronate complex* collected at the ethanol-water interface. The complex was collected and recrystallized from water (yield, 30 mg. or 0·1% of dose). It had m.p. and mixed m.p. 125–128° with the corresponding product from aniline urine. (Found: N, 9·6. $C_{20}H_{31}O_7N_3$ requires N, 9·9%.) It gave a characteristically rapid Tollens reaction and reduced Benedict's solution readily on warming.

## DISCUSSION

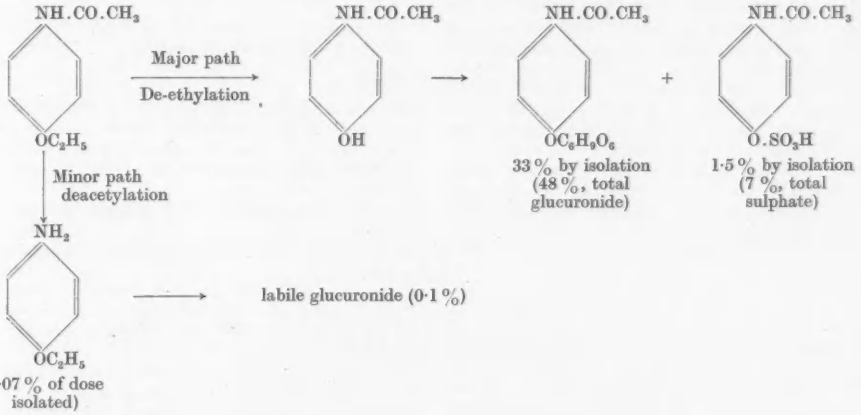
The present work shows that the main metabolites of phenacetin in the rabbit are the same as those of acetanilide, i.e. *p*-acetamidophenylglucuronide and *p*-acetamidophenylsulphuric acid, and quantitatively these account for 54% (47% as glucuronide and 7% as ethereal sulphate) of the phenacetin fed. These metabolites are produced in roughly the same ratio as found in *p*-acetamidophenol or acetanilide metabolism, for the glucuronide/ethereal sulphate ratio for phenacetin is 6·9, for *p*-acetamidophenol, 6·3 and for acetanilide, 5·8 (see Smith & Williams, 1948). From our present results and those on acetanilide it appears that phenacetin and acetanilide owe their therapeutic activity to their metabolic conversion to *p*-acetamidophenol (cf. Hinsberg & Treupel, 1894). This phenol is probably then inactivated by conjugation with glucuronic and sulphuric acids. In the case of acetanilide the process of activation involves oxidation, whereas with phenacetin it involves de-ethylation thus:




Phenacetin is more satisfactory as an analgesic and antipyretic drug than acetanilide. It has been suggested that the rate at which a drug of this group

is metabolized (presumably to *p*-acetamidophenol) depends on its molecular size (Hinsberg & Treupel, 1894). Thus molecules smaller than phenacetin are rapidly metabolized and are consequently more toxic, whereas with larger molecules such as the propyl and amyl analogues of phenacetin, the change is probably slower and, while toxic effects are less marked, there is also a loss of therapeutic effect (see Gaddum, 1944).

we have not yet elucidated. These glucuronides are relatively easy to detect for they readily decompose yielding free glucuronic acid which forms a characteristic crystalline complex in the presence of *p*-toluidine and ammonium ions. This complex was isolated from both phenacetin and acetanilide urines, the yields corresponding to 0·1 % of the dose in each case. It was suggested in an earlier paper (Smith & Williams, 1948) that the metabolism of

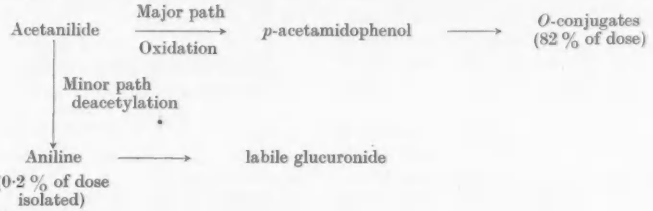



If it is conceded that both the therapeutic and toxic effects of acetanilide and phenacetin are due to the metabolic formation of *p*-acetamidophenol, then the greater efficiency and lesser toxicity of phenacetin are due to its de-ethylation being a slower process than the oxidation of acetanilide. The toxic effects of phenacetin are also less than those of acetanilide because the slower rate of production of *p*-acetamidophenol permits a more efficient detoxication by conjugation.

The extent of deacetylation of phenacetin in the rabbit is very small, but we were able to detect traces of phenetidine in the urine. This small amount of phenetidine should give rise to phenetidine metabolites. Similarly, acetanilide is slightly deacetylated by the rabbit, and should, therefore, give rise to small amounts of aniline metabolites. Now the major metabolites of both aniline and phenetidine in the rabbit are labile glucuronides whose structure acetanilide would be similar to that of aniline in animals capable of extensively deacetylating the aromatic acetamido group. Such animals are the dog, cat, and pigeon (Krebs, Sykes & Bartley, 1947). As mentioned earlier Hinsberg & Kast (1887) observed that a dog receiving phenacetin excreted a strongly reducing urine. This observation can now be explained, for the dog probably converts phenacetin to a considerable extent to phenetidine which may then give rise to the labile glucuronide which we have found to be the major metabolite of phenetidine in the rabbit.

The main features of the metabolisms of phenacetin in the rabbit are as shown above. These findings are probably applicable to man but not to the pigeon, dog and cat.

In the case of acetanilide, a minor pathway of metabolism can now be added to the results given earlier (Smith & Williams, 1948):



SUMMARY

1. The fate of phenacetin has been studied in the rabbit and it has been found to be largely transformed into *p*-acetamidophenylglucuronide and *p*-acetamidophenylsulphuric acid, these occurring in the urine in the ratio 6·9 : 1. The major metabolic change undergone by phenacetin is, therefore, de-ethylation, followed by conjugation.

2. Deacetylation of phenacetin takes place only to a very minor extent. This was shown by the detection of traces of free *p*-phenetidine in the urine, and the isolation of minute amounts of a crystalline complex of *p*-toluidine and ammonium glucuronate derived from a labile glucuronide which is a major metabolite of *p*-phenetidine in the rabbit.

3. Acetanilide is also slightly deacetylated, for acetanilide urine also contains traces of a labile glucuronide which is a major metabolite of aniline (see Smith & Williams, 1949*a*).

4. The results obtained have been correlated with the known therapeutic and toxic effects of phenacetin and acetanilide.

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# Studies in Detoxication

## 23. THE FATE OF ANILINE IN THE RABBIT

BY J. N. SMITH AND R. T. WILLIAMS
*Department of Biochemistry, University of Liverpool*

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In the past numerous investigations (see summary by Gross, 1946) have been carried out on the fate of acetanilide in the body, but few on aniline. This is probably the result of the belief that aniline and acetanilide are interconvertible *in vivo*. We have now found that in the rabbit this is not wholly true. In this animal acetanilide is almost entirely converted into the glucuronide and ethereal sulphate of *p*-acetamidophenol, the excretion of compounds containing free diazotizable amino groups being about 6–7 % of the dose (Smith & Williams, 1948*a*). Furthermore, *p*-substituted acetanilides are either excreted completely unchanged or deacetylated only to a very small extent (Smith & Williams, 1948*b*, 1949*a*). In this paper we shall show that aniline gives rise to metabolites different from those of acetanilide.

The recent work of Krebs, Sykes & Bartley (1947) has shown that the extent of deacetylation of the aromatic acetamido group depends on animal species, deacetylation being extensive in the cat, dog and pigeon, but very small in man and the rabbit. From this it follows that aniline and acetanilide are likely to give rise to similar metabolites in animals such as the dog and cat, but to different ones in man and the rabbit.

Earlier work on the fate of aniline appears to be very scanty. Müller (1887) studied a human case of poisoning by 25 g. of aniline, and found that the urine reduced Fehling's solution and contained conjugated *p*-aminophenol. Schmiedeberg (1878) fed dogs with aniline acetate and identified *p*-aminophenol in the urine after acid hydrolysis. According to Elson, Goulden & Warren (1946) rats probably excrete aniline as the ethereal sulphate of *p*-aminophenol. It is clear from this earlier work that conjugated *p*-aminophenol is a metabolite of aniline, but we shall show that in the rabbit this phenol is not a major metabolite.

The study of the metabolic fate of aniline is not only of considerable theoretical interest, but it is also important because of the possible role of aniline as a bladder carcinogen (for discussion see Goldblatt, 1947).

### I. QUANTITATIVE INVESTIGATION OF TYPES OF COMPOUNDS EXCRETED

#### EXPERIMENTAL

*Glucuronic acid and ethereal sulphate* in urine were determined as described in earlier papers from this laboratory (e.g. Smith & Williams, 1948*a*).

*Diazo test.* Throughout this paper this term means diazotization with 1% $NaNO_2$ and dilute HCl, then addition of 1% ammonium sulphamate followed by coupling with 1% ethanolic 1-naphthyldimethylamine.

*Free aromatic amino groups* were determined using a modification of the Bratton & Marshall (1939) procedure for sulphanilamide. Three compounds, namely, aniline hydrochloride and *o*- and *p*-aminophenylglucuronides (Williams, 1943) were used as standards. If a dilute solution of any of these three compounds is diazotized and coupled with *N*-(1-naphthyl)ethylenediamine as in the Bratton & Marshall procedure a red (aniline and *o*-aminophenylglucuronide) or red-purple (*p*-aminophenylglucuronide) colour develops gradually and reaches a stable intensity in 24 hr. A similar slow rate of colour development was also found when 1-naphthyldimethylamine and sulphatoethyltoluidine were used as coupling agents, but they were not as sensitive as naphthylethylenediamine. In order to obtain reproducible results diazotization had to be prolonged for 10 min. at 20° before adding the coupling agent, and furthermore it was found that the final colour intensity depended on the concentration of acid in the solution, a point which had to be considered in the construction of calibration curves for 'free' and 'total' amino groups.

The procedure finally adopted was as follows. The urine was diluted so as to contain an equivalent of 1–8 mg./l. of aniline: 10 ml. of diluted urine were mixed with 0·5 ml. 4N-HCl followed by 1 ml. 0·1% $NaNO_2$ and allowed to stand 10 min. at room temperature, then 1 ml. of 0·5% ammonium sulphamate was added followed 2 min. later by 1 ml. of 0·1% aqueous *N*-(1-naphthyl)ethylenediamine hydrochloride. After mixing, the solution was left for 24 hr., then made up to 25 ml. with water, and the colour measured with a Spekker photoelectric absorptiometer using an Ilford no. 605 yellow-green filter. The readings were then referred to calibration curves constructed in the same way, but using standard aqueous solutions of aniline hydrochloride, *o*- or *p*-aminophenylglucuronide instead of urine. The *o*-glucuronide gives the same red colour as does aniline the colour ratio *o*-glucuronide/aniline being 1·04. The *p*-glucuronide gives a slightly different colour (red-purple), and using the same filter (no. 605) the colour ratio *p*-glucuronide/aniline = 0·84. In this procedure the free *o*- and *p*-aminophenols do not yield interfering colours, and if present in urine are not estimated.

'Total' aromatic amino groups were estimated by the same general procedure. In this case 10 ml. of diluted urine were mixed with 1·5 ml. 4N-HCl and heated for 1 hr. in a boiling water bath. After cooling the solution, the procedure was as indicated above, except that the absorptiometer readings were referred to calibration curves constructed using 1·5 ml. 4N-HCl instead of 0·5 ml. By this procedure acetanilide in pure solution could be determined almost quantitatively. Under the hydrolysis conditions used, *o*-aminophenylglucuronide is only hydrolyzed to a slight extent, for the colour ratio *o*-aminophenylglucuronide/aniline changes only from 1·04 to 0·91. With *p*-aminophenylglucuronide hydrolysis of the glycuronidic link proceeds further for the colour ratio *p*-aminophenylglucuronide/aniline changes from 0·84 to 0·59.

Owing to the variable extents of hydrolysis of the conjugated metabolites encountered in the urines examined, the estimations of 'total' aromatic amino groups are not easily interpreted, and, therefore, are quoted (in Table 1) with reserve. The estimations of 'free' aromatic amino groups, however, are more capable of interpretation.

## RESULTS

The excretion of conjugated glucuronic acid and ethereal sulphates and of diazotizable amino groups by rabbits receiving oral doses of aniline, phenetidine, *o*- and *p*-aminophenols and their *N*-acetyl derivatives are given in Table 1.

The results for the acetylated compounds are more easily interpreted than those for the free amino compounds because the qualitative results (Smith & Williams, 1948*a*, 1949*a*) show that the metabolism of the acetyl derivatives is the less complicated; they do not undergo deacetylation to any great extent.

For the acetyl compounds the percentage of total diazotizable amino groups (Table 1, column 7) is expected to be only slightly less than the percentage of glucuronide (column 3), for the following reason.

Table 1. *The excretion of conjugated glucuronic acid and sulphate and of diazotizable amino groups by rabbits receiving aniline and its derivatives orally (averaged results)*

| | | Percentage excreted | | | | |
|---|---|---|---|---|---|---|
| | | | | | Containing diazotizable amino groups | |
| Compound fed | Dose (mg./kg.) | As glucuronide (*G*) | As ethereal sulphate (*E*) | *G*/*E* | Free | After acid hydrolysis |
| Aniline | 200 | 70 | 28 | 2·5 | 40* | 60* |
| *p*-Ethoxyaniline (phenetidine) | 300 | 120 | 30 | 4·0 | 3† | 25† |
| *p*-Aminophenol | 250 | 43 | 18 | 2·5 | 28† | 45† |
| *o*-Aminophenol | 210 | (28)‡ | 25 | 1·1 | 33§ | 42§ |
| Acetanilide | 250 | 70 | 12 | 5·8 | 6,* 7† | 37,* 61† |
| *p*-Ethoxyacetanilide (phenacetin) | 350 | 48 | 7 | 6·9 | 0† | 49† |
| *p*-Acetamidophenol | 280 | 63 | 10 | 6·3 | 0† | 49† |
| *o*-Acetamidophenol | 280 | 39 | 20 | 2·0 | 3·5§ | 33§ |

* Aniline as standard. † *p*-Aminophenylglucuronide as standard.
‡ This figure will be too low because *o*-aminophenylglucuronide cannot be estimated by the naphthoresorcinol method.
§ *o*-Aminophenylglucuronide as standard.

Qualitative results show that the main glucuronide excreted after administration of acetanilide, phenacetin and *p*-acetamidophenol is *p*-acetamidophenylglucuronide and the ethereal sulphate is *p*-acetamidophenylsulphuric acid. Under the conditions of hydrolysis for the estimation of total amino groups the ethereal sulphate is completely hydrolyzed to *p*-aminophenol which gives no colour in the estimation procedure; the glucuronide, however, is deacetylated to *p*-aminophenylglucuronide which is only slightly hydrolyzed to *p*-aminophenol. Thus the amount of *p*-aminophenylglucuronide produced by hydrolysis, and estimated by diazotization and coupling should be only slightly less than the amount of *p*-acetamidophenylglucuronide estimated by the naphthoresorcinol reaction in the unhydrolyzed urine.

An examination of the figures for *p*-aminophenol suggests that both *p*-acetamido- and *p*-aminophenylglucuronide are its metabolites, because the figure for the total diazotizable amino groups (45 %) is almost identical with the figure for glucuronide output (43 %), and there is an increase in diazotizable amino groups on acid hydrolysis from 28 to 45 %. This increase must be due to hydrolysis of the *p*-acetamido- to the *p*-amino-glucuronide, because hydrolysis of the ethereal sulphate will give *p*-aminophenol which has no effect on the diazo reaction.

In the case of *o*-aminophenol, the total glucuronide formed is probably more accurately given by the figure for the total amino groups (42 %); the figure of 28 % obtained by the naphthoresorcinol method is definitely too low because Hanson, Mills & Williams (1944) have shown that *o*-aminophenylglucuronide is not completely hydrolyzed under the conditions of their method for estimating glucuronic acid.

## II. THE ISOLATION OF ANILINE METABOLITES

### Experimental

#### 1. *The effects of aniline on rabbits*

Each dose (*c.* 0·5 g./kg. in most cases) was dissolved in 20 ml. water containing just enough HCl to give a clear solution. The amount of HCl used was less than that required to form the hydrochloride and this solution was better tolerated than aniline hydrochloride. Single doses of 1·3 g./kg. were not fatal in this form, but doses of 0·3 g./kg. repeated daily for 4 days were fatal. Aniline had no narcotic effect similar to that of acetanilide (Smith & Williams, 1948*a*).

#### 2. *The nature of aniline urine*

The urine had a dark colour but a normal pH of 8. This colour did not appear to be due to porphyrins, for the urine under ultraviolet light showed not a red but a pale green fluorescence. The urine readily reduced Benedict's solution and the reducing substance was not glucose (osazone test). No reducing substance could be extracted by ether, butanol or amyl alcohol at different values of pH.

The urine from two rabbits, each of which had received 3 g. of aniline, was found to contain 4·4 g. extra glucuronic acid (estimated by the method of Hanson *et al.* 1944). By titration with Benedict's quantitative reagent the urine was found to contain 3·8 g. of reducing material, calculated as glucuronic acid. From this it can be deduced that aniline urine contains at least two glucuronides, one reducing and present in large amount, and the other non-reducing. The urine gave a strong red colour in the diazo test thus showing the presence of free aromatic amino groups. The Tollens test for glucuronic acid was given very readily, for a deep blue precipitate was formed even before the mixture had reached boiling point. These tests suggested that aniline urine may contain a very labile, reducing glucuronide. Neither the urine itself nor ether extracts of it gave any colour with $FeCl_3$ and from this it could be concluded that free aminophenols were not present. The urine was slightly laevorotatory; filtered and clarified with a few drops of HCl it gave $\alpha_D$ −0·18° in a 1 dm. tube. From a knowledge of the extra glucuronic acid content (0·8 g./100 ml.) of this urine and assuming that the glucuronide present is *p*-aminophenylglucuronide $\alpha_D$ for 1 dm. should be −0·64° (see Williams, 1943). Thus the conclusion can be drawn that the main glucuronide is not that of *p*-aminophenol.

#### 3. *Extraction of aniline urine with ether. Isolation of unchanged aniline*

(*a*) *At acid reaction.* Continuous ether extraction of acidified aniline urine yielded no material derived from aniline.

(*b*) *At alkaline reaction.* A 24 hr. urine (1200 ml.), collected after feeding 12 g. aniline, was treated with a few ml. of 2N-KOH and extracted continuously with ether for 1 hr. The extract, dried over $Na_2SO_4$, was evaporated, leaving 0·42 g. of a dark oil which was identified as aniline by preparing from it tribromoaniline (m.p. and mixed m.p. 117–118°) and benzanilide (0·76 g.; m.p. and mixed m.p. 160°). In other experiments the dried ether extract was treated with a saturated ethereal solution of oxalic acid and the crystalline precipitate of aniline hydrogen oxalate (m.p. 158°; Anselmino (1903) gives m.p. 163°) collected, dried and weighed. The recoveries of free aniline in five experiments were 3, 5, 6, 9 and 9·5 % (average 6·5 %) of the dose. Neither acetanilide nor aminophenol was found.

(*c*) *The origin of the free aniline.* The aniline isolated could either be free, unchanged aniline or it could arise by decomposition of a labile precursor. That the second possibility was unlikely was shown as follows: 12 g. of aniline were fed to six rabbits and the urine (950 ml.) collected for 24 hr. To 350 ml. of the urine were added 10 g. of $Na_2CO_3$ and the whole was extracted with ether for 4 hr. On addition of ethereal oxalic acid to this extract there was obtained 0·8 g. of aniline hydrogen oxalate. Another 350 ml. of the urine was treated in a similar manner except that, before ether extraction, the alkaline urine was boiled vigorously for 10 min. The yield of aniline hydrogen oxalate was again 0·8 g. In another experiment 400 ml. of aniline urine were made alkaline and exhaustively extracted with ether to remove free aniline. To the urine were now added 40 ml. of conc. HCl and the whole was boiled for 20 min. The hydrolyzed urine was cooled, neutralized with 40 % NaOH,

made alkaline with solid $Na_2CO_3$ and extracted exhaustively with ether. Although this extract contained aminophenols (see below) it contained no trace of aniline.

These experiments show that the aniline present in the urine probably occurs as such for there is no increase in the amount present when the urine is heated with acid or alkali.

4. *The ethereal sulphate fraction of aniline urine. Isolation of* o- *and* p-*aminophenols and detection of* 4-*aminoresorcinol*

(*a*) *Mild hydrolysis of aniline urine.* Aniline urine (400 ml. from 5·8 g. aniline) was made alkaline with a little 2N-NaOH and the free aniline removed with ether as described above. It was then acidified with 40 ml. conc. HCl and boiled for 20 min. After cooling, it was neutralized with NaOH, made alkaline with 1 g. solid $Na_2CO_3$ and continuously extracted with ether for 1·5 hr. The extract was reduced to 10 ml. and the crystals (60 mg.) which separated collected: they had m.p. 180°, gave a purple colour with $FeCl_3$ and were identified as *p*-aminophenol by conversion to *ON*-dibenzoyl-*p*-aminophenol, m.p. and mixed m.p. 230° (yield, 0·9% of the dose). The filtrate was reduced to 0·5 ml. on the water bath and on cooling 50 mg. of yellow plates separated. These were collected and found to give a red colour with $FeCl_3$ and an intense yellow colour with nitrous acid, but no immediate red colour in the diazo test. They were identified as *o*-aminophenol by conversion into *ON*-dibenzoyl-*o*-aminophenol, m.p. and mixed m.p. 178–179° (yield, 0·7% of the dose). The residue, which remained after separation of the *o*- and *p*-aminophenols, gave colour reactions for 4-aminoresorcinol, for on shaking in air in the presence of NaOH it gave an intense blue colour which on standing faded to green and finally (1 hr.) to brown (Henrich & Wagner, 1902).

We prepared 4-aminoresorcinol hydrochloride by the method of Henrich & Wagner (1902) and carried out trial separations of the three phenols, but we failed to isolate the labile 4-aminoresorcinol from aniline urine in pure crystalline form. When fed to a rabbit, 4-aminoresorcinol (0·5 g.) causes the excretion of a urine which is almost black in colour.

(*b*) *Acetone-ammonium sulphate fractionation of aniline urine.* A total of 8 g. aniline was fed to four rabbits. The 24 hr. urine (800 ml.) was evaporated *in vacuo* at 40–45° to 100 ml. The concentrate was acidified with a little HCl, saturated with $(NH_4)_2SO_4$ and then shaken with 250 ml. acetone. The ethereal sulphates present, together with some glucuronides, passed into the acetone layer. The main bulk of the labile glucuronide (see p. 247) and other material reacting with naphthoresorcinol remained in the aqueous layer. The acetone extract was made alkaline with solid $K_2CO_3$ and concentrated to a small volume *in vacuo* at 40–50°. Dry acetone was now added and a small flocculent glucuronide-containing precipitate which rapidly turned into gum was deposited on standing. After removal of this gum, the acetone solution gave only a weak naphthoresorcinol reaction and contained no inorganic sulphate, but gave strong tests for ethereal sulphate. It was taken to small bulk *in vacuo*. Attempts to crystallize the organic sulphate or prepare crystalline derivatives such as benzylamine salts were unsuccessful. The concentrate gave strong tests for free amino groups and ethereal sulphates, but did not reduce ammoniacal $AgNO_3$. It was, therefore, acidified with 0·1 vol. conc. HCl and boiled for 10 min. to hydrolyze the ethereal sulphates and then diluted to 50 ml. with water. The solution now reduced ammoniacal $AgNO_3$ and gave an intense blue colour on shaking in air with NaOH solution, indicating the presence of 4-aminoresorcinol. The main bulk of the hydrolyzed solution was extracted with $2 \times 100$ ml. ether. Evaporation of the ether left a small amount of dark tar which gave no diazo or $FeCl_3$ test. The residual solution was, therefore, made alkaline with solid $Na_2CO_3$ and extracted with $2 \times 100$ ml. ether. From this extract *p*-aminophenol (3·1% of the dose) and *o*-aminophenol (0·3% of the dose) were isolated and identified as before.

The residue after separation of these two phenols left on evaporation a small tarry residue giving a faint red colour in the diazo test. On shaking the tar in air with NaOH solution an intense blue colour was obtained which faded to brown in about an hour. 4-Aminoresorcinol was thus present, but attempts to obtain crystalline derivatives were unsuccessful.

5. *The glucuronide fraction of aniline urine*

(*a*) *Preparation of the glucuronide gum.* 12 g. of aniline were fed to eight rabbits and the urine was collected for 24 hr. Free aniline (3% of the dose) was removed with ether as described in section 3 (*b*). The alkaline urine was now made faintly acid and lead acetate added until no further precipitation occurred. The precipitate was removed and the filtrate was neutralized with ammonia and an excess of saturated basic lead acetate added. The precipitate was filtered, washed with water, and then suspended in water and Pb removed with $H_2S$. The Pb-free filtrate was dried *in vacuo* at 40–50° to a reddish gum (24 g.) which was purified by dissolving in the minimum of water and pouring into ethanol. A precipitate of inorganic material separated and after filtration the solution was concentrated *in vacuo* at 40–50° to a gum which reduced Benedict's solution on warming, gave a weak red colour in the diazo test, and reacted very rapidly in the Tollens test for glucuronic acid. The gum could not be induced to crystallize and its potassium, brucine, *o*-toluidine and benzylamine salts separated from ethanol as flocculent precipitates which readily formed gums. It contained no ether-soluble material.

(*b*) *Isolation of* p-*aminophenol from the gum.* The purified glucuronide gum obtained after feeding 5 g. of aniline was dissolved in 150 ml. 5N-$H_2SO_4$, filtered and then boiled for 2·5 hr. The dark hydrolysate, after cooling and making alkaline, was extracted for 3 hr. with ether which on evaporation left a crystalline residue from which 150 mg. (5% of the dose) of *p*-aminophenol, m.p. 182–184°, were obtained. No other phenol was identified in the hydrolysate.

(*c*) *Methylation and acetylation of the gum. Isolation of* p-*acetamidophenyltriacetylglucuronide methyl ester.* Methylation of the gum with dimethyl sulphate and alkali followed by $Ag_2O$ and methyl iodide yielded no crystalline product. A similar result was obtained on acetylation of the gum with pyridine and acetic anhydride. A crystalline derivative was, however, obtained by the following procedure: 2 g. of the gum dissolved in 10 ml. absolute ethanol were treated with an excess of diazomethane in 100 ml. dry ether; a small precipitate appeared. After standing for 24 hr. at room temperature the precipitate had partly dissolved again

and the whole was evaporated *in vacuo*. The product, a brown gum which would not crystallize, was dissolved in 30 ml. of a pyridine-acetic anhydride mixture (1:1) and kept overnight. It was then poured into 200 ml. water and the solution was extracted with chloroform. The extract, washed free of acetic acid with water, gave on evaporation a gum which crystallized from acetone-water mixtures as long needles, m.p. 100° (resolidifying and then melting again indefinitely at 170–180°). This compound appears to be a dimorphic *p*-acetamidophenyltriacetylglucuronide methyl ester (yield 70 mg.; 1% of the dose). On recrystallization from absolute ethanol it yielded needles, m.p. 205°, not depressed by an authentic sample (Smith & Williams, 1948*a*). It showed $[\alpha]_D^{20°} - 22{\cdot}4°$ (*c*, 4 in chloroform). (Found: C, 54·3; H, 5·5. Calc. for $C_{21}H_{25}O_{11}N$: C, 54·0; H, 5·4%.) In another experiment 0·6% of the dose was isolated as this ester.

The two methyl esters, m.p.'s 100 and 205°, have been described by us in an earlier paper (Smith & Williams, 1948*a*) and we suggested that the former was a hydrate of the latter, but we only quoted analyses for the latter compound. We now find that both give analyses correct for the anhydrous compound and show identical specific optical rotations in chloroform. The ester of m.p. 100° gave the following analysis: Found: C, 53·6; H, 5·65; N, 3·2. Calc. for $C_{21}H_{25}O_{11}N$: C, 54·0; H, 5·4; N, 3·0%. $[\alpha]_D - 22{\cdot}1°$ (*c*, 7 in chloroform). We conclude, therefore, that we have here a case of dimorphism. Neither of the two forms obtained from the aniline gum depressed the m.p.'s of the corresponding authentic forms prepared synthetically from *p*-aminophenylglucuronide.

Our results so far indicate that the gum contains either *p*-aminophenylglucuronide or *p*-acetamidophenylglucuronide, or both, but the small yields of the above derivatives suggest that these glucuronides are not major metabolites.

(*d*) *Isolation of* p-*aminophenylglucuronide*. The evidence presented on the nature of the glucuronide gum suggests that it contains at least two glucuronides, viz. large amounts of a labile, reducing glucuronide and the non-reducing *p*-aminophenylglucuronide. Since the ethereal sulphate fraction contains *o*-aminophenol, it is also possible that the gum may contain *o*-aminophenylglucuronide. Attempts were, therefore, made to fractionate the gum.

At first advantage was taken of the fact that the aminophenylglucuronides have isoelectric points where they show minimum solubility. It was found that the *o*-compound had a minimum solubility at *c.* pH 3·0–3·5 and the *p*-compound at *c.* pH 4·0–4·5. Thus the *p*-compound should crystallize best at about pH 4·0–4·5.

Aniline urine (140 ml.) clarified with 50 ml. colloidal iron was evaporated *in vacuo* to 70 ml. then buffered to pH 4·3 and seeded with *p*-aminophenylglucuronide. No precipitate appeared after 5 days at 0°.

The basic lead acetate precipitate from aniline urine after feeding 10 g. of aniline was prepared in the usual manner and Pb removed with $H_2S$. The filtrate (350 ml.) from the PbS had pH 2·3. A 50 ml. portion of this filtrate was brought to pH 4·3 with acetate buffer, but no precipitate was obtained after 2 days at 0°. The rest of the filtrate (300 ml.) was made faintly alkaline with ammonia and then treated with cold saturated mercuric acetate solution. A small pinkish precipitate separated and was filtered off. The labile glucuronide appeared in the filtrate together with the main bulk of the naphthoresorcinol-reacting material of the urine. The mercuric acetate precipitate did, however, contain a glucuronide. It was suspended in water and Hg removed with $H_2S$. The filtrate from the HgS was evaporated *in vacuo* at 40–50° to 15 ml. and this was brought to pH 4·3 with 0·5 M-Na acetate. The mixture was kept at 0° overnight. The crystalline precipitate (180 mg., or 0·6% of the dose) was filtered off, washed with water, ethanol and then ether. It was recrystallized from hot water, from which it formed felted needles, m.p. 215–216°, not depressed by authentic *p*-aminophenylglucuronide (Williams, 1943). It showed $[\alpha]_D^{20°} - 83{\cdot}4°$ (*c*, 4·2, in 0·5 N-$H_2SO_4$). (Found: C, 48·2; H, 5·6; N, 4·6. Calc. for $C_{12}H_{15}O_7N.H_2O$: C, 47·5; H, 5·6; N, 4·6%.) In another experiment, using 12 g. aniline, 295 mg. (0·8% of the dose) of the glucuronide were isolated.

This experiment was repeated in exactly the same manner, except that the glucuronide gum solution was saturated with $SO_2$ prior to the Hg precipitation. The yield of *p*-aminophenylglucuronide was not increased, nor was it increased by boiling the urine with hot alkali prior to preparation of the gum. These results indicate that *p*-aminophenylglucuronide occurs in the urine as such and is not derived from a labile glucuronide.

(*e*) *Isolation of* p-*acetamidophenylglucuronide*. The glucuronide fraction from the urine (350 ml.) of four rabbits which had collectively received 8 g. of aniline was prepared as before. The fraction was concentrated to 30 ml. of syrupy liquid to which was added, with shaking, 200 ml. of absolute ethanol. A reducing gum was precipitated which yielded 7 g. (15% of the dose) of the crystalline *p*-toluidine-ammonium glucuronate complex (see p. 247). The ethanolic solution after removing the gum was now reduced *in vacuo* to 10 ml., and again precipitated with ethanol to remove further traces of the labile glucuronide. The concentration and precipitation with ethanol was repeated once more. In this way most of the labile glucuronide was removed and the final ethanolic solution was concentrated at 40–50° to a non-reducing gum (2 g.) which contained conjugated glucuronic acid, but only gave a diazo test after hydrolysis by acid. The non-reducing gum was now dissolved in 50 ml. 95% ethanol and the solution treated with 1 g. of benzylamine followed by 400 ml. ethyl acetate. After 1 hr. at 0° the crystalline precipitate (200 mg., 0·5% of the dose) was collected and recrystallized twice from ethanol and ethanol-ethyl acetate. The crystals were identified as the benzylamine salt of *p*-acetamidophenylglucuronide, m.p. and mixed m.p. 195–198°, $[\alpha]_D^{15°} - 59°$ (*c*, 0·7 in water) (see Smith & Williams, 1948*a*). A further quantity of this salt was obtained from the mother liquor, but it could not be satisfactorily purified.

We have attempted to assess by various means the amount of *p*-acetamido- and *p*-amino-phenylglucuronides in aniline urine. By actual isolation we found 0·8% of the dose as *p*-amino- and at least 0·5% as *p*-acetamidophenylglucuronide, and by isolation of the acetylated methyl ester, 1% of the dose. These values are obviously minimal. A study of the ultraviolet absorption spectrum of the gum (see p. 247) gave an estimate of 10%. The difference between the 'extra' glucuronic acid content of the acid content of the urine and its reducing power towards Benedict's reagent gave a value of 15%. We can, therefore, conclude that about 10–15% of the aniline fed is excreted as free and acetylated *p*-aminophenylglucuronide.

(*f*) *The isolation of free* D-*glucuronic acid from the gum.* It was mentioned in § 2 that aniline urine had strong reducing properties, Benedict's and Fehling's solution being very readily reduced on warming. Furthermore, the reducing substance was not glucose. The reducing substance appeared in the basic lead acetate precipitate of the urine, and constituted the major fraction of the glucuronide gum prepared from this precipitate. On examination of the gum it was found possible to isolate, without the use of hydrolytic methods, considerable amounts of free glucuronic acid. The significance of this free glucuronic acid is not yet clear to us.

The isolation of glucuronic acid was achieved through the use of *p*-toluidine. Glucuronic acid forms in the presence of *p*-toluidine and $NH_4^+$ ions a sparingly soluble, crystalline compound whose components are two molecules of *p*-toluidine, and one of ammonium glucuronate. It appears to be a convenient compound for isolating free glucuronic acid, but it is not formed from the lactone, glucurone. The preparation of authentic samples of this compound, and of the corresponding compound from D-galacturonate together with a discussion of their nature are described in the succeeding paper (Smith & Williams, 1949*b*).

The isolation of the compound from aniline urine was achieved as follows. A solution of 8 g. of the purified glucuronide gum (see § 5 (*a*)) dissolved in 5 ml. cold water was mixed with a suspension of 4 g. *p*-toluidine in 1 ml. ethanol and 4 ml. warm water. The toluidine dissolved on stirring, and, on cooling, the mixture crystallized. After keeping at 0° for a few hours the crystals were filtered at the pump, washed with a little ethanol followed by a large volume of ether (yield, 7 g.: this corresponds to 21% of the dose of aniline, assuming that one molecule of aniline gives rise to one of glucuronic acid). The *p-toluidine-ammonium glucuronate complex*, recrystallized from hot dilute ethanol, formed colourless rectangular plates, m.p. 125–128° (decomp.), $[\alpha]_D^{20°} + 20° \rightarrow +11°$ (constant value in *c.* 3 hr.) (*c*, 5 in 0·4 N-HCl). Ultraviolet absorption spectrum: in water, $\lambda_{max.}$ 234 mμ., $\epsilon_{max.}$ 16,900, $\lambda_{max.}$ 285 mμ., $\epsilon_{max.}$ 2700; in N-HCl, $\lambda_{max.}$ 264 mμ., $\epsilon_{max.}$ 400. Elementary analysis suggested that two hydrates of this complex existed (cf. the hydrates of glucose toluidide; Irvine & Gilmour, 1909). The one less frequently obtained contained 0·5 $H_2O$ more than the other. (Found: (1) C, 55·5; H, 7·2; N, 9·6. $C_{20}H_{29}N_3O_6 . 1{\cdot}5H_2O$ requires: C, 55·3; H, 7·4; N, 9·7%. (2) C, 56·7; H, 7·2; N, 9·25; glucuronic acid, 47·6. $C_{20}H_{29}N_3O_6 . H_2O$ requires C, 56·45; H, 7·3; N, 9·9; glucuronic acid, 45·7%.) The compound quickly reduced Benedict's reagent on warming and gave the Tollens test for glucuronic acid very rapidly. With Nessler's reagent it gave a brown precipitate, a reaction not given by *p*-toluidine.

(*g*) *Spectroscopic observations on the glucuronide gum.* The isolation of free glucuronic acid from the gum raises the question of its source. It could arise from a labile glucuronide of aniline metabolism or aniline may stimulate the excretion of free glucuronic acid. We thought that spectroscopic examination of the gum might give us information on the first of these alternatives. A possible glucuronide would be the unknown β-phenylhydroxylamine glucuronide. This compound would probably be labile and break up to phenylhydroxylamine which would rapidly form azoxybenzene. The latter can be detected by its characteristic ultraviolet absorption. When fed to rabbits, β-phenylhydroxylamine (200 mg.) does not cause the excretion of a reducing urine.

The glucuronide gum for spectroscopic examination was obtained by working up the 24 hr. urine of a rabbit which had received 2 g. aniline (see § 5 (*a*)). It was dissolved in 2·5 l. 50% aqueous ethanol and the solution examined with a Hilger E 3 spectroscope. The absorption spectra of the gum solution, *p*-aminophenylglucuronide, *p*-acetamidophenylglucuronide and azoxybenzene are reproduced in Fig. 1.

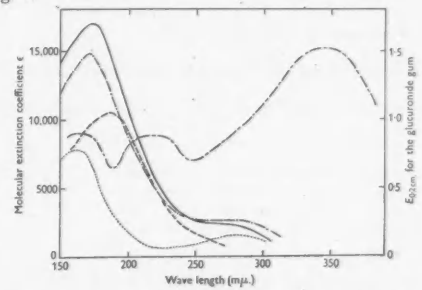



Fig. 1. The ultraviolet absorption spectrum of the glucuronide gum from aniline urine compared with the spectra of possible components. –·–·–·, the glucuronide gum (after feeding 2 g. aniline) dissolved in 2·5 l. 50% aqueous ethanol: $\lambda_{max.}$ 236 mμ., $E_{0·2cm.} = 1·5$; — — —, *p*-acetamidophenylglucuronide in water: $\lambda_{max.}$ 244 mμ., $\epsilon_{max.}$ 10,800; ·····, *p*-aminophenylglucuronide in 0·1 N-KOH: $\lambda_{max.}$ 231 mμ., $\epsilon_{max.}$ 7700 and $\lambda_{max.}$ 291 mμ., $\epsilon_{max.}$ 1600; ————, sum of the absorptions of *p*-acetamido- and *p*-aminophenylglucuronides; — · — ·, azoxybenzene in ethanol: $\lambda_{max.}$ 235 mμ., $\epsilon_{max.}$ 9000; $\lambda_{max.}$ 249 mμ., $\epsilon_{max.}$ 8900; $\lambda_{max.}$ 324 mμ., $\epsilon_{max.}$ 15,200.

The gum solution showed a band at $\lambda_{max.}$ 236 mμ. and an inflexion at 285 mμ. There was no significant absorption at 324 mμ., in which region azoxybenzene absorbs strongly. It can, therefore, be concluded that the gum contains no azoxybenzene, and consequently that phenylhydroxylamine is unlikely to be present (cf. the cases of sulphanilamide and 4:4′-disulphonamidoazoxybenzene studied by Williams (1946) and of 4-hydroxylamino-2:6-dinitrotoluene and 2:2′:6:6′-tetranitro-4:4′-azoxytoluene in TNT metabolism studied by Channon, Mills & Williams (1944)). The peak at 236 mμ. may be due to *p*-amino- and *p*-acetamido-phenylglucuronide, for if the curves for these compounds are summated, the resultant curve has the same shape as that of the gum. This argument is only valid if irrelevant absorption at the shorter wavelengths is assumed to be negligible. The glucuronide gum may contain, on these grounds, *p*-amino- and *p*-acetamido-phenylglucuronide in roughly equal amounts.

## Results

The results of the work on isolation of aniline metabolites are summarized in Table 2.

## DISCUSSION

The present work shows that the metabolism of aniline in the rabbit is a more complicated process than one would have imagined at first sight and, although a number of the metabolites have been identified, what we think is a major metabolite has,

Table 2. *Derivatives isolated from the urine of rabbits receiving aniline orally*

| Derivative | M.p. | $[\alpha]_D$ | Yield as % of dose |
|---|---|---|---|
| (*a*) Unconjugated: | | | |
| Aniline hydrogen oxalate | 158° | — | 6–9 |
| Benzanilide | 160° | — | 3–9 |
| (*b*) From glucuronide fraction: | | | |
| *p*-Toluidine complex of ammonium glucuronate | 125–128° | +21° (in 0·4 N-HCl) | 21 |
| *p*-Aminophenylglucuronide | 215–216° | −83·4° (in 0·5 N-$H_2SO_4$) | 0·8 |
| Benzylamine salt of *p*-acetamidophenylglucuronide | 195–198° | −59° (in water) | 0·5 |
| *p*-Acetamidophenyltriacetylglucuronide methyl ester | 205° and 100° | −22·4° (in chloroform) | 1 |
| *p*-Aminophenol (by hydrolysis) | 182–184° | — | 5 |
| (*c*) From ethereal sulphate by hydrolysis: | | | |
| *p*-Aminophenol | 183° | — | 3 |
| *o*-Aminophenol dibenzoate | 176–179° | — | 0·3 |
| 4-Aminoresorcinol (detected) | — | — | ? |

so far, eluded our efforts to isolate and identify it. In what follows we shall attempt to assess, in various ways, the amounts of each metabolite excreted.

The isolation experiments account for nearly 40 % of the aniline fed (see Table 2). This figure is made up as follows: free and acetylated *p*-aminophenylglucuronide, 5 % (based on the isolation of *p*-aminophenol from the glucuronide gum); free aniline, up to 9 % (see § 3 (*b*)); *p*-aminophenylsulphuric acid, 3·1 %; *o*-aminophenylsulphuric acid, 0·7 % (from hydrolysis of the ethereal sulphate fraction, see §§ 4 (*a*) and (*b*)). Assuming that each molecule of glucuronic acid isolated as the *p*-toluidine complex is derived from a labile glucuronide generated from one molecule of aniline, then 21 % of the aniline fed is excreted as a labile glucuronide (§ 5 (*f*)).

The excreted free diazotizable amino groups correspond to 40 % of the aniline fed (Table 1). The compounds which could contribute to this figure are free aniline (6·5 %, see § 3 (*b*)), *p*-aminophenylglucuronide (5–8 %, § 5 (*d*) and (*g*)) and *p*-aminophenylsulphuric acid (*c.* 28 %). *o*-Aminophenylsulphuric acid probably does not contribute because Burkhardt & Wood (1929) have shown that when diazotized it loses its sulphate group yielding *o*-aminophenol.

If it is assumed that one molecule of aniline gives rise to one of glucuronide, then 70 % of the amine is excreted as conjugated glucuronic acids (Table 1). The isolation experiments indicate that aniline urine may contain three glucuronides, i.e. *p*-amino- and *p*-acetamido-phenylglucuronides and a 'labile glucuronide'. The amount of the first two was assessed at 10–15 % and, therefore, about 55–60 % of the aniline fed may be excreted as the labile glucuronide. We can do little more than speculate at present on the nature of the labile glucuronide.

Aniline urine is reducing and free glucuronic acid in large amounts can be isolated from it as the *p*-toluidine-ammonium glucuronate complex. Now this free glucuronic acid could occur in the urine as such or be produced by the breakdown of a labile glucuronide. The excretion of free glucuronic acid as such, appears to us, at the moment, to be unlikely though not impossible. The existence of a labile glucuronide, however, is feasible on the following grounds. Less than 50 % of the aniline fed can be accounted for as diazotizable compounds; the rest may be changed in the body to a non-diazotizable form which is the aglycone of a labile glucuronide. A dihydrohydroxyaniline glucuronide (I) could account for some of the facts. Such a structure as (I) contains an aliphatic amino group, a new asymmetric carbon atom—which could account for the low optical rotation of the urine—and an aldehyde-ammonia group—which would make it labile and release ammonia and glucuronic acid to form the *p*-toluidine-ammonium glucuronate complex. When this complex is formed from the acidic glucuronide gum no external ammonia is necessary, yet when it is formed from pure glucuronic acid ammonia must be added. Structure (I) is that of a hexa-1:4-diene

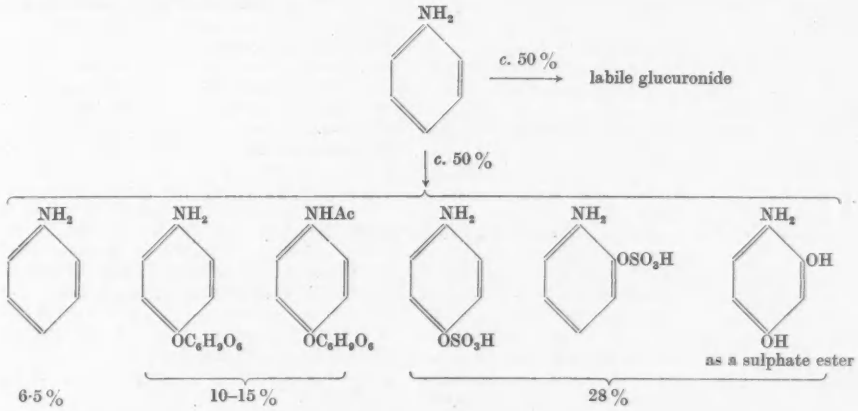
(I)

and therefore should show little or no light absorption. It will be recalled that ultraviolet absorption spectrum of the glucuronide gum appears to be that of glucuronides of *p*-acetamido- and *p*-amino-phenol only.

We have also proved that aniline is slightly acetylated *in vivo* by the isolation of *p*-acetamidophenylglucuronide, but no acetanilide was detected. It is possible that here aniline is first oxidized to

*p*-aminophenol which is then acetylated and *O*-conjugated; there may be no direct acetylation of aniline.

It is clear that the metabolism of aniline is very complex. On available evidence we suggest the following tentative scheme:




Three aminophenols were found in the urine. The qualitative experiments on the ethereal sulphate fraction suggest that *p*-aminophenol preponderates. *o*-Aminophenol and 4-aminoresorcinol were found only in the ethereal sulphate fraction. The only phenol isolated from the glucuronide fraction was *p*-aminophenol, but we cannot eliminate the possibility that the *o*-compound is also present in this fraction, for Hanson *et al.* (1944) have shown that *o*-aminophenylglucuronide is very resistant to acid hydrolysis. We feel, however, that the *o*-glucuronide is not present in appreciable amounts because although it is more easy to isolate from urine (Williams, 1943) than its *p*-isomer, we isolated only the latter from the glucuronide gum.

The detection of 4-aminoresorcinol shows that the *o*- and *p*-aminophenols derived from aniline undergo further oxidation to a trisubstituted benzene. Porteous & Williams (1949) have already shown that benzene gives rise to a trisubstituted compound, hydroxyquinol, in the rabbit.

We suggested in an earlier paper (Smith & Williams, 1948*a*) that biological oxidation of an aromatic ring takes place at those carbon atoms which possess a certain minimum of electronic activation. On this basis aniline should be oxidized *in vivo* in the *o*- and *p*-positions and this is in agreement with our findings (cf. acetanilide, Smith & Williams, 1948*a*).

## SUMMARY

1. The metabolic fate of aniline in the rabbit has been studied.

2. About 28 % of the dose is excreted as the ethereal sulphates of *o*- and *p*-aminophenol and 4-aminoresorcinol. The *o*- and *p*-aminophenols were isolated.

3. About 70 % of the aniline fed is excreted as glucuronides, two of which were isolated and identified as *p*-acetamido- and *p*-amino-phenylglucuronide. These two account for 10–15 % of the aniline fed.

4. It is suggested that the main metabolite of aniline is a labile glucuronide. This substance accounts for more than 50 % of the aniline fed and may be a reduced aniline derivative. It also accounts for the reducing properties of aniline urine.

5. The labile glucuronide readily breaks up to give free glucuronic acid, which has been isolated as a crystalline *p*-toluidine-ammonium glucuronate complex.

6. No evidence was found to support the view that aniline is converted to phenylhydroxylamine *in vivo*.

7. Aniline gives rise to excretion of small amounts of *N*-acetyl derivatives. Whether or not aniline is directly acetylated has not been proved.

8. Aniline is oxidized in the *o*- and *p*-positions and the significance of this finding is discussed.

9. The metabolites of aniline in the rabbit are not the same as those of acetanilide. The significance of this is discussed.

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# Studies in Detoxication

## 24. THE METABOLISM OF *p*-PHENETIDINE (*p*-ETHOXYANILINE) WITH SOME OBSERVATIONS ON THE ANISIDINES (METHOXYANILINES)


BY J. N. SMITH AND R. T. WILLIAMS
*Department of Biochemistry, University of Liverpool*


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This study of the fate of alkoxyanilines in the rabbit was pursued because we wanted to know the effect of alkoxyl groups on the biological acetylation of the amino group in compounds of the type $R.C_6H_4NH_2$. The study became of further interest when it was found that administration of *p*-phenetidine and the *o*-, *m*- and *p*-anisidines resulted in the excretion of reducing urines as in the case of aniline (Smith & Williams, 1949*b*).

Little previous work has been done on *p*-phenetidine; Edlefson (1900) showed that, in man, it gave rise to conjugated *p*-aminophenol and Elson, Goulden & Warren (1946) suggested that it behaved similarly in the rat.

### EXPERIMENTAL

*Methods.* The quantitative estimations of the excretion of glucuronic acid, ethereal sulphate, and diazotizable amino groups in the urine of rabbits receiving *p*-phenetidine hydrochloride orally are quoted in the preceding paper (Smith & Williams, 1949*b*).

Phenetidine was fed as the hydrochloride, m.p. 234°.

#### *The isolation of phenetidine metabolites*

##### (1) *The nature of phenetidine urine*

After feeding phenetidine in doses of 0·2–0·5 g./kg., rabbits excreted a brown urine (pH *c.* 8) similar in appearance to aniline urine. It reduced Benedict's reagent and gave a very rapid naphthoresorcinol reaction. It gave a permanganate colour with $FeCl_3$ and a positive diazo reaction.

The urine was optically active. The urine from two rabbits, each of which had received 2 g. of phenetidine hydrochloride, was estimated to contain 4·7 g. of extra glucuronic acid. A 10 ml. portion of this urine was clarified with dialyzed iron and made up to 20 ml. In a 2 dm. tube $\alpha_D$ was $-0{\cdot}42°$, whilst normal rabbit urine treated in the same way has no detectable rotation. If the glucuronide in the urine were that of either *p*-aminophenol or *p*-acetamidophenol, the expected $\alpha_D$ would be *c.* $-1{\cdot}3°$. Thus it follows that the glucuronides from phenetidine cannot be entirely *p*-aminophenol derivatives.

##### (2) *Isolation of unchanged phenetidine*

The filtrate from the preparation of the basic lead acetate fraction of phenetidine urine (see p. 251) was made alkaline with KOH, filtered and continuously extracted with ether for 4 hr. The bases in the ether were extracted with 2 N-HCl. This solution was made alkaline and extracted in a funnel with ether. This ethereal solution was dried over NaOH for 24 hr., filtered, and saturated with dry HCl gas. The crystalline precipitate of phenetidine hydrochloride (m.p. and mixed m.p. 234°) was filtered off. The yields of unchanged phenetidine in these experiments were 1·5% of the dose at a level of 0·5 g./kg. and 2 and 5% at 0·8 g./kg.

##### (3) *The ethereal sulphate fraction*

(*a*) *Isolation of 2-hydroxy-4-ethoxyaniline (hydroxyphenetidine).* The 24 hr. urine (1250 ml.) after feeding 18 g. phenetidine hydrochloride was concentrated at 40° *in vacuo* to 250 ml., acidified with dilute HCl, saturated with $(NH_4)_2SO_4$ and extracted with $2 \times 250$ ml. acetone. The acetone extract was made alkaline with solid $K_2CO_3$, concentrated *in vacuo* to 100 ml., treated with 1 l. dry acetone, filtered and concentrated to 50 ml. The concentrate was now extracted five times with an equal volume of ether to remove free phenetidine (recovered as the hydrochloride, m.p. 230°). (Found: C, 55·1; H, 6·9; N, 7·6. Calc. for

$C_8H_{11}ON.HCl$: C, 55·3; H, 7·0; N, 8·0%.) Further concentration of the extracted solution caused the separation of urea and some inorganic material which were removed by shaking with 95% ethanol and filtering. The filtrate, now containing largely ethereal sulphates with some glucuronide, was treated with 300 ml. absolute ethanol which threw out the glucuronide as a non-reducing flocculent precipitate (600 mg.) which we were unable to identify. The ethanolic solution was now reduced *in vacuo* to a syrup (30 ml.), which at this stage gave negative tests for glucuronic acid, but positive ethereal sulphate and diazo tests. However, we were unable to isolate the sulphate or a derivative in crystalline form. Therefore 10 ml. of the syrup were dried *in vacuo*, and the residue boiled for 5 min. with 5 ml. conc. HCl and 20 ml. of 5% aqueous $BaCl_2$. After cooling, the $BaSO_4$ was filtered off and the filtrate treated with a little $Na_2S_2O_4$ which removed some of the dark brown colour. Then it was made alkaline with $K_2CO_3$, stirred with 5 ml. acetic anhydride for 10 min., and extracted with 20 ml. chloroform. The extract was dried over $CaCl_2$ and allowed to evaporate at room temperature. A sticky mass of crystals separated which became readily filterable on addition of ether. Recrystallization from benzene gave rectangular plates (170 mg., 2·5% of the dose) of 2-acetoxy-4-ethoxyacetanilide, m.p. and mixed m.p. 127–129°. (Found: $OC_2H_5$, 19·8. Calc. for $C_{12}H_{15}O_4N$: $OC_2H_5$, 19·0%.)

(*b*) *Synthesis of 2-acetoxy-4-ethoxyacetanilide.* 2-Hydroxy-4-ethoxyaniline hydrochloride, prepared according to Henrich & Birkner (1913), yielded with aqueous $Na_2CO_3$ and acetic anhydride *2-acetoxy-4-ethoxyacetanilide*, m.p. 127–129° after recrystallization from benzene.

A more convenient method of synthesis starts from 4-nitrosoresorcinol 1-ethyl ether, which was prepared according to the method of Henrich & Rhodius (1902) for the methyl ether. 4-Nitrosoresorcinol 1-ethyl ether (0·5 g.) was reduced with 2 g. of Sn in 5 ml. conc. HCl. The reduction mixture was evaporated on the water bath to remove excess HCl, neutralized with $NaHCO_3$ and extracted with ether to remove 2-hydroxy-4-ethoxyaniline. The extract was treated with an excess (10 ml.) of acetic anhydride and evaporated *in vacuo* to a small volume. The residue was mixed with an excess of $Na_2CO_3$ solution, and the whole shaken until the oil, which had separated, crystallized. The *2-acetoxy-4-ethoxyacetanilide* (0·27 g.) was dried and recrystallized from benzene. It formed rectangular plates, m.p. 127–129°. (Found: C, 60·5; H, 6·05; N, 6·1; $OC_2H_5$, 19·4. $C_{12}H_{15}O_4N$ requires C, 60·7; H, 6·4; N, 5·9; $OC_2H_5$, 19·0%.)

### (4) *The glucuronide fraction*

(*a*) *Detection of a glucuronide of* p*-aminophenol.* The basic lead acetate precipitate of a 24 hr. urine (340 ml.) from two rabbits, each of which had received 2 g. of *p*-phenetidine hydrochloride, was prepared in the usual manner. It was suspended in water and Pb removed with $H_2S$. The lead-free filtrate was concentrated *in vacuo* to a brownish gum (5 g.) which had properties similar to that obtained from aniline urine. Part of this gum (4·6 g.) was treated with 150 ml. of ethereal diazomethane (from 5 g. nitrosomethylurea). The ether was removed and the neutral tarry residue dissolved in a mixture of 20 ml. pyridine and 20 ml. acetic anhydride. After 18 hr. at room temperature the mixture was diluted with 100 ml. water and extracted with 3 × 20 ml. chloroform. After washing with dilute acid and alkali and drying over $CaCl_2$, the chloroform extract was evaporated, leaving a partially crystalline gum (0·94 g.). This product was dissolved in hot ethanol and on cooling to 0°, 150 mg. (1·5% of the dose) of *p*-acetamidophenyltriacetylglucuronide methyl ester (m.p. and mixed m.p. 205–207°); $[\alpha]_D^{15°}$ − 23·5° (*c*, 6·1 in chloroform) separated (Smith & Williams, 1948, 1949*b*).

Isolation of this compound showed that the glucuronide fraction contained either *p*-aminophenylglucuronide or its *N*-acetyl derivative or both.

(*b*) *Isolation of* p*-acetamidophenylglucuronide.* The glucuronide fraction was prepared by lead acetate treatment from the 24 hr. urine of 6 rabbits which had collectively received 12 g. phenetidine hydrochloride. This fraction was concentrated so that 50 ml. of an aqueous syrup was obtained. This was now diluted with 400 ml. absolute ethanol which was added with shaking. A gum was thrown out and the ethanol (*A*) removed by decanting. The gum was dissolved in 10 ml. water and on treating this with 5 g. *p*-toluidine in 5 ml. ethanol and cooling to 0° overnight, 8 g. of the *p*-toluidine-ammonium glucuronate complex (m.p. 125°) crystallized and was filtered off.

The ethanolic solution *A* was now evaporated to 20 ml. and again diluted to 250 ml. with ethanol to throw out a further portion of insoluble gum. Again the ethanol was decanted and the process repeated. The insoluble gum obtained yielded a further 1 g. of *p*-toluidine-ammonium glucuronate complex. The mother liquor, after removal of the insoluble gum, was now taken to dryness *in vacuo*. The residual gum (5 g.) gave no diazo reaction until hydrolyzed: 4·7 g. of it were dissolved in 50 ml. 95% ethanol, and 3 ml. benzylamine were added. The mixture was diluted to 500 ml. with ethyl acetate. From this solution there was obtained 2 g. (6·4% of the dose) of the benzylamine salt of *p*-acetamidophenylglucuronide, m.p. and mixed m.p. 195–198° (after recrystallization from 95% ethanol), $[\alpha]_D^{25°}$ − 59° (*c*, 7·8 in water). (Found: N, 6·5. Calc. for $C_{21}H_{26}O_8N_2.H_2O$: N, 6·2%.)

(*c*) *Attempted isolation of* p*-aminophenylglucuronide.* *p*-Aminophenylglucuronide was isolated from aniline urine from the mercuric acetate fraction (see Smith & Williams, 1949*b*, preceding paper). An attempt was made to isolate it from phenetidine urine by a similar procedure. We thought that it might occur in phenetidine urine in small amounts, but its isolation as such or as a benzylamine salt was not achieved.

(*d*) *Spectroscopic observations on the glucuronide fraction.* As in the case of aniline, the absorption spectrum of the glucuronide fraction of *p*-phenetidine urine can be accounted for by the presence of relatively small amounts of aminophenylglucuronides. An aqueous solution (400 ml.) of the glucuronide fraction, prepared, after feeding 8·75 g. phenetidine, as in section 4 (*a*) above, was estimated (naphthoresorcinol method) to contain 7·1 g. of glucuronic acid. It was made faintly alkaline with ammonia, diluted 100 times with water and its ultraviolet absorption measured in a Hilger E3 spectroscope. It had a maximum extinction ($E_{0·3\ cm.}$) at 240 mμ. of 1·2. The form of the absorption curve closely resembled that of *p*-acetamidophenylglucuronide (Smith & Williams, 1948) and the presence of *p*-aminophenylglucuronide was not indicated. If it be accepted that the absorption was due to *p*-acetamidophenylglucuronide, then it can be calculated from the value of $E_{0·3\ cm.}$ that 4·9 g. of it is present, i.e. 2·9 g. or 41% of the total glucuronic acid.

### (5) *The isolation of free glucuronic acid from the urine of rabbits receiving alkoxyanilines orally*

(*a*) p-*Phenetidine.* The glucuronide gum from the urine of twelve rabbits which had collectively received 24 g. of *p*-phenetidine hydrochloride was prepared (see section 4 (*a*)). From this the *p*-toluidine-ammonium glucuronate complex was prepared as described in the preceding paper on aniline (Smith & Williams, 1949*b*). The yield was 34 g. or 58% of the dose assuming that one molecule causes the excretion of one of glucuronic acid. It was recrystallized (plates) from hot aqueous ethanol. (Found: C, 56·55; H, 7·4; N, 10·7; $OC_2H_5$, 0; glucuronic acid, 47·6; *p*-toluidine, 48; $H_2O$, 4·5. $C_{20}H_{29}O_6N_3.H_2O$ requires C, 56·45; H, 7·3; N, 9·9; $OC_2H_5$, 0; glucuronic acid, 45·6; *p*-toluidine, 50·4; $H_2O$, 4·2%.) It decomposed at 125–128° and showed $[\alpha]_D^{20°}$ $-60° \xrightarrow{24\text{ hr.}} +2·5°$ (constant value) (*c*, 0·5 in 1:3 ethanol-water), $[\alpha]_D^{20°}$ $+18 \rightarrow +11°$ (constant) (*c*, 5·2 in 0·4 N-HCl).

The complex was analyzed for *p*-toluidine by heating on the water bath for 0·5 hr. with 0·5 N-HCl, cooling, then diazotizing and coupling with naphthylethylenediamine. The diazo colour was measured in a Spekker absorptiometer and compared with a standard calibration curve constructed with pure *p*-toluidine hydrochloride.

The water content of the complex could not be determined directly by drying to constant weight because it decomposed with loss of toluidine. An approximate estimate of the water content of the sample analyzed above was obtained using Fischer's reagent (pyridine, $I_2$ and $SO_2$; see Fischer, 1935). As mentioned in the preceding paper, we had reason to believe that this compound formed two hydrates similar to the glucose *p*-toluidides of Irvine & Gilmour (1909). Our experiments suggested that the hydrate containing $1·5H_2O$ was formed when crystallization was carried out at 0° (see section 6 (*b*)) and the one containing $1H_2O$ was formed at room temperature or higher. When the sample above was recrystallized at 0° it gave C, 55·9; H, 7·05; N, 9·6. $C_{20}H_{29}O_6.1·5H_2O$ requires C, 55·3; H, 7·4; N, 9·7%.

When the compound was dissolved in cold 20% NaOH it gave off ammonia (proved by aeration into picric acid solution, to give ammonium picrate, m.p. and mixed m.p. 270° (decomp.)). In the Van Slyke apparatus the compound released $N_2$ in 0·5 hr. equivalent to 3·2% N; the first formula quoted above requires 3·3% N as $NH_4$.

(*b*) o-*Anisidine.* *o*-Anisidine (3·6 g., b.p. 218–220°) was dissolved in the minimum of dilute HCl and the solution diluted to 40 ml. Two rabbits each received 20 ml. of this solution. It had a slight narcotic effect but the animals were easily roused by handling. One rabbit died 5 days later. A reducing urine was excreted even during the first 2 hr. after feeding. The 24 hr. urine (380 ml.) was brown and darkened further on standing. The colour was discharged by $Na_2S_2O_4$. The urine reduced Fehling's solution, gave a strong red diazo reaction and an intense Tollens test. The glucuronide gum (3 g.) was prepared as for phenetidine urine and, on treatment with *p*-toluidine as before, 1·3 g. (equivalent to 14% of the anisidine fed) of the *p*-toluidine-ammonium glucuronate complex (m.p. 125–128° decomp.) was obtained; $[\alpha]_D^{20°}$ $+18°$ (initial value) (*c*, 3·9 in 0·4 N-HCl). Absorption spectrum: $\lambda_{max.}$ 234 m$\mu$., $\epsilon_{max.}$ 16,800 in water. (Found: C, 56·0; H, 7·3; N, 9·6%.)

(*c*) p-*Anisidine.* Two rabbits were each fed with 2 g. of *p*-anisidine (m.p. 57°) in 20 ml. of water containing the minimum amount of dilute HCl. The urine was reducing, and the glucuronide gum (3 g.), prepared as before, yielded 1 g. (equivalent to 9·5% of the dose) of the *p*-toluidine-ammonium glucuronate complex (m.p. 125–128° decomp.), $[\alpha]_D^{20°}$ $+20°$ initial (*c*, 5·4 in 0·4 N-HCl). (Found: C, 56·25; H, 7·1; N, 8·8%.)

(*d*) m-*Anisidine.* In a similar fashion the toluidine complex was obtained after feeding *m*-anisidine (b.p. 246–247°/720 mm.). It had m.p. 125–128° decomp., $[\alpha]_D^{20°}$ $+19 \rightarrow +15°$ (*c*, 5 in 0·4 N-HCl) and spectral absorption $\lambda_{max.}$ 234 m$\mu$., $\epsilon_{max.}$ 16,600 and $\lambda_{max.}$ 285 m$\mu$., $\epsilon_{max.}$ 2600 in water.

### (6) *Synthesis of* p-*toluidine-ammonium uronate complexes*

(*a*) D-*glucurone.* When *p*-toluidine in ethanol is added to an aqueous solution of glucurone containing a little ammonia, no *p*-toluidine complex separates.

(*b*) D-*glucuronic acid.* D-Glucurone (0·5 g.) was converted into barium glucuronate by treatment with 1 equiv. of 0·3 N-$Ba(OH)_2$ at room temperature for 3 hr.; 0·5 ml. of saturated $(NH_4)_2SO_4$ solution was added and the mixture filtered from $BaSO_4$. To the filtrate was added 0·6 g. *p*-toluidine in 5 ml. ethanol and then the mixture was diluted to 50 ml. with ethanol. After 24 hr. the crystals (0·54 g., 45% of theory) of the *p-toluidine-ammonium glucuronate* complex were filtered off and washed with water and ethanol. It was recrystallized from ethanol-water and cooled to 0°. Rectangular plates were obtained, m.p. 125–130° (decomp.), $[\alpha]_D^{20°}$ $-59° \xrightarrow{24\text{ hr.}} +2°$ (constant value) (*c*, 0·3 in 1:3 ethanol-water). (Found: C, 55·3; H, 7·4; N, 9·7. $C_{26}H_{29}N_2O_6.1·5H_2O$ requires 55·3; H, 7·4; N, 9·7%.) Ultraviolet absorption in water: $\lambda_{max.}$ 234 m$\mu$., $\epsilon_{max.}$ 16,800 and $\lambda_{max.}$ 285 m$\mu$., $\epsilon_{max.}$ 2600. It was insoluble in ethanol and organic solvents and sparingly soluble in water. It reduced Benedict's reagent readily and gave a rapid Tollens test for glucuronic acid. It was identical in all respects with the compound obtained from the alkoxyaniline urines.

The same complex was obtained from crude barium glucuronate prepared by acid hydrolysis of Turkey gum.

(*c*) *From* (−)-*Menthyl-*D-*glucuronide.* The complex (m.p. 123° decomp.) was obtained in poor yield (7%) from an acid-hydrolyzed solution of menthylglucuronide. (Found: C, 56·3; H, 7·2; N, 9·4% ($[\alpha]_D$ $+27 \rightarrow +15°$) (*c*, 2·7 in 0·4 N-HCl).)

(*d*) *The complex from* D-*galacturonic acid.* When an aqueous solution of D-galacturonic acid is mixed with an equal volume of an ethanolic solution of *p*-toluidine, no apparent change takes place. But as soon as the mixture is treated with a few drops of ammonia solution (sp.gr. 0·88) it crystallizes completely. The *p-toluidine-ammonium* D-*galacturonate complex*, obtained in excellent yield, forms plates, m.p. 135–139° (decomp.) and $[\alpha]_D^{20°}$ $-31 \rightarrow +5°$ (constant value after 24 hr.) (*c*, 1·2 in 50% aqueous ethanol). Its absorption spectrum is similar to that of the glucuronic acid analogue; in water it shows $\lambda_{max.}$ 232 m$\mu$., $\epsilon_{max.}$ 16,600; $\lambda_{max.}$ 285 m$\mu$., $\epsilon_{max.}$ 2850. (Found: C, 56·5; H, 7·3; N, 9·5. $C_{20}H_{29}O_6N_3.H_2O$ requires C, 56·45; H, 7·3; N, 9·9%.) This was slightly more soluble than the glucuronic acid complex.

## DISCUSSION

It is clear that the metabolism of *p*-phenetidine is a complex problem which requires further investigation. So far we have proved that it undergoes (*a*) de-ethylation, (*b*) acetylation, and (*c*) oxidation without de-ethylation.

The occurrence of de-ethylation and deacetylation was proved by the isolation of *p*-acetamidophenylglucuronide, which could be formed in two ways as follows:

(i) phenetidine → phenacetin
or (ii) phenetidine → *p*-aminophenol
→ *p*-acetamidophenol → glucuronide.

The first of these mechanisms appears to be the most likely, for if *p*-aminophenol were formed we would have expected to isolate its glucuronide, but we did not. For phenetidine the difference between the free and total diazotizable amino groups amounted to 22% (see Table 1 of the preceding paper, Smith & Williams, 1949*b*). This figure, together with a consideration of the spectrum of the glucuronide gum, suggests that about one quarter of the phenetidine fed is excreted as compounds containing the acetamido group.

It was shown (Smith & Williams, 1949*a*) that the de-ethylation of phenacetin *in vivo* was virtually complete, the main excretion products being *O*-conjugates of *p*-acetamidophenol. With phenetidine, however, there is only a partial de-ethylation, for we isolated from the urine 2-hydroxy-4-ethoxyaniline in which the original ethoxyl group of phenetidine is intact. It appears, therefore, that breaking of the ether linkage *in vivo* takes place more readily if the *p*-amino group is acetylated. The isolation of 2-hydroxy-4-ethoxyaniline is also of interest in relation to the orientation of the hydroxyl group entering the phenetidine molecule. Both the $NH_2$ and $OC_2H_5$ groups are *o*-*p*-directing (ring activating), but the oxidation takes place *o*- to the amino group. The amino group is known to be chemically more strongly directing than the alkoxyl group (Fieser & Fieser, 1944) and the isolation of 2-hydroxy-4-ethoxyaniline fits in with this view.

Rabbits receiving phenetidine excrete ethereal sulphates corresponding to 30% of the dose. Hydrolysis of the sulphate fraction yielded a reddish tar consisting largely of phenolic substances, from which 2-hydroxy-4-ethoxyaniline was isolated in yields of 8–9% of the ethereal sulphate fraction (i.e. 2·5% of the dose). We failed to detect *p*-aminophenol in this fraction, but there were suggestions that 4-aminoresorcinol was present. It appears, therefore, that the main phenol in the sulphate fraction is 2-hydroxy-4-ethoxyaniline.

The exact nature of the glucuronide fraction of phenetidine urine has not been solved, although it is clear from this work that it presents, as in the case of aniline, a new aspect of the metabolism of aromatic amines. On measuring the 'extra' glucuronic acid excretion it was found that more than one molecule of glucuronic acid was excreted/molecule of phenetidine fed. The average glucuronide output was 120% of the dose. This high figure could mean that a metabolite carrying two glucuronic acid molecules is excreted. Alternatively, it may mean that phenetidine stimulates the excretion of free glucuronic acid. The *p*-toluidine complex of glucuronic acid was isolated in large amounts, equivalent to 58% of the phenetidine fed, and we believe that a considerable proportion of phenetidine is excreted as a labile glucuronide. A minor component of the glucuronide fraction is *p*-acetamidophenylglucuronide, which was isolated. Although we isolated *p*-aminophenylglucuronide from aniline urine, it was not found in phenetidine urine, a fact which is supported by the very low amount (3% of the dose) of free diazotizable amino groups found in phenetidine urine.

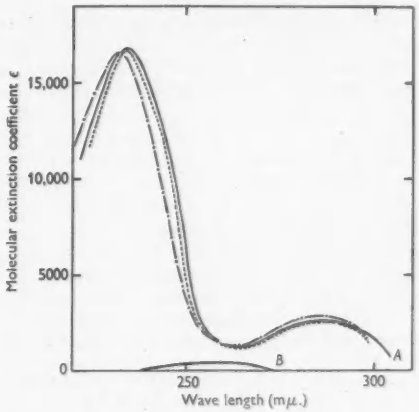
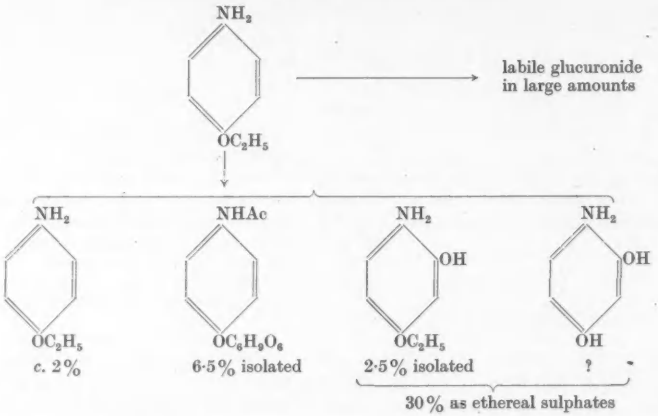



Fig. 1. Ultraviolet absorption spectra of *p*-toluidine-ammonium uronate complexes. —— *A*, *p*-Toluidine-ammonium D-glucuronate complex from aniline urine in water: $\lambda_{max.}$ 234 mμ., $\epsilon_{max.}$ 16,900 and $\lambda_{max.}$ 285 mμ., $\epsilon_{max.}$ 2700; *B*, same as *A* but in N-HCl: $\lambda_{max.}$ 261, $\epsilon_{max.}$ 400; -------- synthetic *p*-toluidine-ammonium D-glucuronate complex (prepared from D-glucuronic acid) in water: $\lambda_{max.}$ 234 mμ., $\epsilon_{max.}$ 16,800 and $\lambda_{max.}$ 285 mμ., $\epsilon_{max.}$ 2600; -·-·-· *p*-toluidine-ammonium D-galacturonate complex (prepared from D-galacturonic acid) in water: $\lambda_{max.}$ 232 mμ., $\epsilon_{max.}$ 16,600 and $\lambda_{max.}$ 285 mμ., $\epsilon_{max.}$ 2850.




The evidence presented here on the fate of phenetidine in the rabbit does not allow us to make very definite conclusions and we suggest the above scheme very tentatively.

*The p-toluidine-glucuronate complex and the labile glucuronides derived from aromatic amines.* The *p*-toluidine-ammonium glucuronate complex has been isolated from *p*-phenetidine (58 % of the dose), aniline (21 %), *o*-anisidine (14 %), *m*-anisidine (12 %) and *p*-anisidine (9·5 %) urines. Only traces of this complex were obtained from acetanilide and phenacetin urines (Smith & Williams, 1949*a*), these traces probably being formed as a result of the production of very small amounts of aniline and phenetidine by deacetylation. It is clear, therefore, that the hypothetical labile glucuronides are only produced from the free aromatic amines and not from the *N*-acetylated amines. Further investigations are in progress on the fate of other types of substituted anilines, and so far we have found that *p*-bromoaniline does not give rise in the rabbit to reducing urines. In the case of the naphthylamines, we have found that the 1-derivative gives rise to a urine which reduces Fehling's but not Benedict's reagent, whereas with the 2-derivative the urine only slightly reduced Fehling's solution.

The *p*-toluidine complex cannot be prepared directly from the 'arylamine urine', but is obtained readily from the glucuronide gums prepared at ordinary temperatures by systematic lead acetate precipitations. In the case of *m*-anisidine the complex is only obtained with difficulty from the glucuronide gum and often only after the addition of a little ammonia. The addition of ammonia is unnecessary in the other cases, e.g. aniline and phenetidine. This strongly suggests that the glucuronic acid is derived from labile glucuronides and that the one from *m*-anisidine has some stability.

The structure of the crystalline *p*-toluidine-ammonium glucuronate complex also requires discussion. From its absorption spectrum (see Fig. 1) it is clear that the only absorbing component is *p*-toluidine (according to Dede & Rosenberg (1934) *p*-toluidine shows two absorption bands in water, at 234 m$\mu$., log $\epsilon$ 3·91 and 288 m$\mu$., log $\epsilon$ 3·15). Its rising mutarotation in ethanol-water ($[\alpha]_D$ $-60 \rightarrow +2°$) suggests that it is a *p*-toluidide (i.e. a *N*-glucuronide) of $\beta$-configuration. On keeping a few weeks the compound turns a light brown. It has no true melting point but decomposes fairly consistently at 125–128°. The analytical data on the compound suggest that it contains 1 molecule of *p*-toluidine, 1 of the *p*-toluidide of ammonium glucuronate and 1 or 1·5 molecules of crystal water. It may be tentatively formulated as

$$CH_3C_6H_4NH_2,\ CH_3C_6H_4NH{-}\underbrace{CH{-}(CHOH)_3{-}CHCOONH_4}_{\text{O}},\ H_2O.$$

D-Galacturonic acid gives the same type of complex whose properties and spectrum (see Fig. 1) are similar to those of the glucuronic acid complex.

## SUMMARY

1. The fate of *p*-phenetidine in the rabbit has been studied, and some observations have been made on the *o*-, *m*- and *p*-anisidines.

2. About 30 % of the phenetidine fed is excreted as ethereal sulphates. The main phenol in the sulphate fraction is 2-hydroxy-4-ethoxyaniline which was characterized as 2-acetoxy-4-ethoxyacetanilide, the synthesis of which is described for the first time. It is suggested that 4-aminoresorcinol is also present.

3. A considerable amount of glucuronide is excreted which appears to be largely a labile glucuronide, the nature of which has not been elucidated.

4. *p*-Acetamidophenylglucuronide has also been isolated from the urine. This substance was obtained in 6 % yields, but may occur in the urine to the extent of 20 % or more.

5. A *p*-toluidine-ammonium glucuronate complex has been isolated from *p*-phenetidine, *o*-, *m*- and *p*-anisidine urines. The yield from phenetidine urine was high.

6. The *p*-toluidine complexes of ammonium glucuronate and galacturonate have been synthesized and studied, and suggestions have been made regarding their nature.

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# A Method for Continuous Graphic Recording of Radioactive Tracer Concentrations from Various Body Regions Simultaneously

By M. REISS, F. E. BADRICK, JEAN M. HALKERSTON and J. H. WHITE
*The Biochemical and Endocrinological Research Department, Bristol Mental Hospitals, Fishponds, Bristol*

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In measuring the changing content of radioactive tracer in different organs of man and animal, it is of great interest to investigate the fate of the tracer at very short time intervals after administration, and also to measure its accumulation simultaneously in several parts of the body. Since information of this kind may not easily be obtained by use of the usual dynatron scalers, certain modifications were necessary to allow continuous recording from different scalers simultaneously.

## EXPERIMENTAL

### *Apparatus*

The internal wiring of the dynatron scaler was slightly modified. The connexions to the internal Post Office meter were diverted to a double pole, double throw, changeover switch (*CH*, Fig. 2), thus enabling the operator to switch over to an external 2-pin socket (*S*). Socket and changeover switch were both mounted on the front panel of the scaler. The 2-pin socket facilitated the connexion with an external Post Office meter (*M*), which had the same resistance (2300 Ω) as the meter mounted inside the scaler. It was possible, by these means, to record every tenth or hundredth impulse on either the internal or external Post Office meter. The latter (Fig. 1, and *M*, Fig. 2) was fitted with a small clamping block (*CB*, Fig. 1) on the moving armature, and in this the stem of the writing point (*WP*) was clamped. A brass rod (*R*) was screwed into the back of the meter, permitting the meter to be mounted on a kymograph, where the impulses were continuously recorded.

The Geiger-Müller counters consisted of tungsten helix γ-tubes, made by 20th Century Electronics Co. Ltd. They were shielded with lead tubes in which a ¼ in. wide longitudinal slot had been made and the tubes clamped in such a way that the slot was placed just over the organ under investigation. The counters were further shielded with black paper, which nullified the photosensitivity of the tubes. An example of the arrangement of the counters, as used in clinical investigations is illustrated in Fig. 3.

For recording the impulses coming from different Geiger-Müller tubes located on various parts of the body, different scalers were employed, each connected with its external counter writer, thus permitting simultaneous recording of fluctuations of tracer concentrations.

### *Clinical investigations*

The assembly described above has been so far used clinically for recording changes in $^{131}$I concentration only. Prior to the injection of $^{131}$I the kymograph was started and the background counts recorded. Then, without stopping the recording mechanism, a dose of $^{131}$I was given.

### *Animal experiments*

The method has proved particularly valuable in recording changes of blood $^{131}$I concentrations.

A length of antioxidant-free 'Telcothene' tubing (obtained from Telegraph Construction and Maintenance Co. Ltd.) of 1·5 mm. bore was wound round a γ-counter tube in the form of a spiral, which was secured by sticking over with transparent adhesive tape. The total volume of this spiral was approximately 4 ml. (see Fig. 4).

In anaesthetized and heparinized rabbits the carotid was exposed, the blood flow interrupted, and cannulae (*C* I and

*C*2) were inserted in the proximal (*CPR*) and distal (*CD*) parts, as shown in Fig. 4. The proximal cannula was first connected with the spiral (*TS*) and the blood allowed to run through until the spiral was full. The free end of the spiral was then connected to the distal cannula avoiding the entry of air bubbles into the system. The T-pieces (*T*1 and *T*2) permitted the taking of samples, the control of flow and, if required, the measurement of blood pressure. The same counter, once prepared, was used for several control experiments. After each experiment, the spiral was cleaned without detachment from the counter tube. After removal from the animal, water was drawn through the spiral by means of a filter pump, followed by washing through with detergent solution; it was finally rinsed again with water and dried.

After the operative procedures were completed, and the background count recorded, $^{131}$I was injected intravenously. The fate of the tracer substance was then recorded from the moment of its entry into the blood stream. The number of impulses could be assessed, for instance, at intervals of 10 sec. In the experiments with which we were concerned, the $^{131}$I uptake of the thyroid was measured at the same time by a *GM* 4 counter enclosed in a lead tube (see Fig. 4).

## RESULTS

Very soon after injection of $^{131}$I into a human subject the thyroid counter began to show an increased activity, whilst changes in the bladder readings occurred later. The gradient of $^{131}$I uptake by the thyroid and other organs was found to vary considerably in different patients. Clinical diagnostic results achieved with this method will be reported later.

The results of a typical animal experiment showing the changes of $^{131}$I in the blood stream and thyroid of a rabbit are illustrated in Fig. 5. It will be noted that about 60 sec. elapse before complete distribution takes place in the blood, and that the concentration rapidly falls to 50% of the maximum value within the first 240 sec. Further, it shows how readily the thyroid takes up iodine, which reaches a high level during the first 30 sec., and then increases more slowly over the following 420 sec.

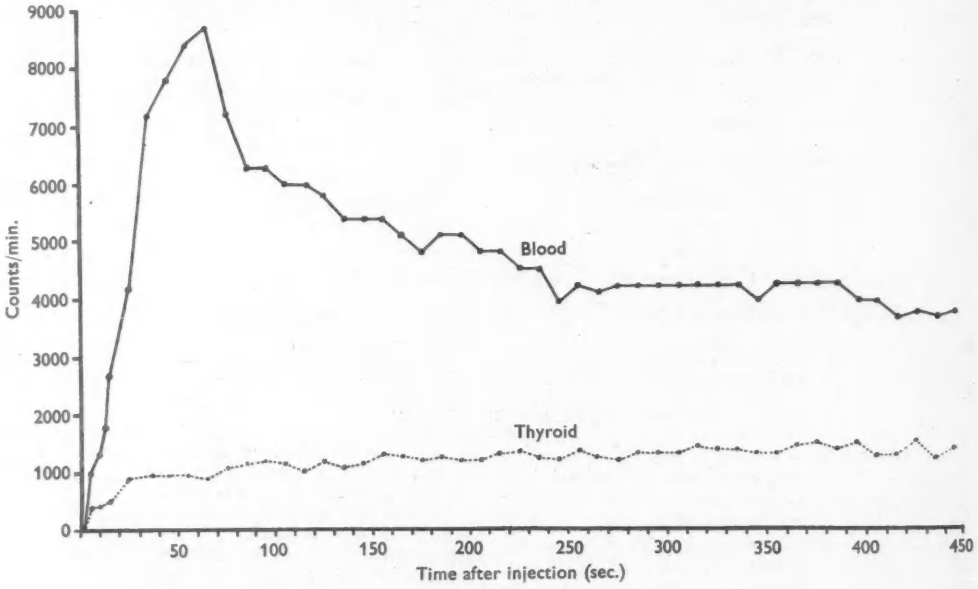


Fig. 5.

The method appears to have considerable heuristic value, and is now used in these laboratories for investigations relating to iodine metabolism.

## SUMMARY

1. A polygraphic device for recording continuous and simultaneous changes of the radioactive tracer concentrations in different body regions is described.

2. Examples of the applicability of the method are described.

3. It was possible, by the use of this method, to study the fate of $^{131}$I from the moment of its entry in the blood stream.

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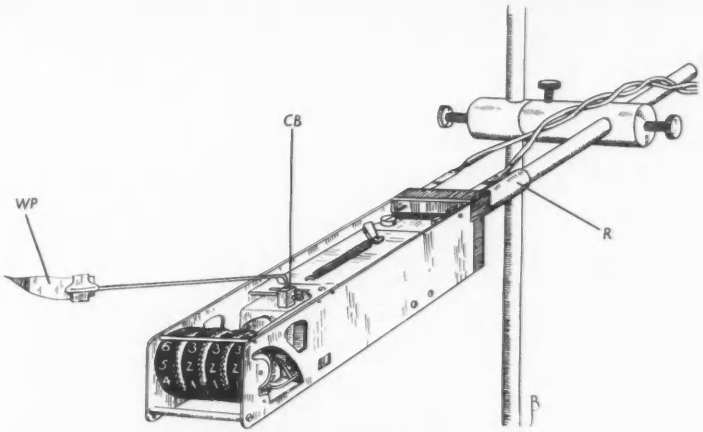


Fig. 1. The counter and writer.

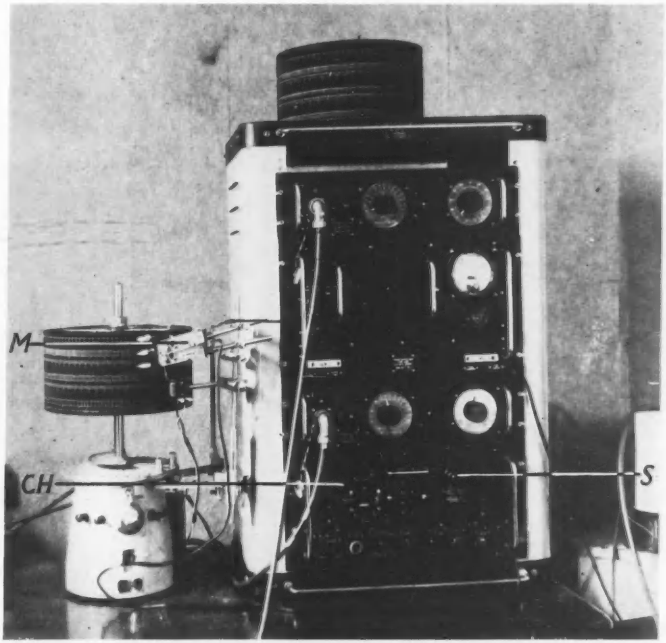


Fig. 2. Showing connexion between scaler and writer.

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Fig. 3. Showing arrangement of counters for simultaneous recording from different parts of the body. 1, thyroid region; 2, liver region; 3, bladder region.

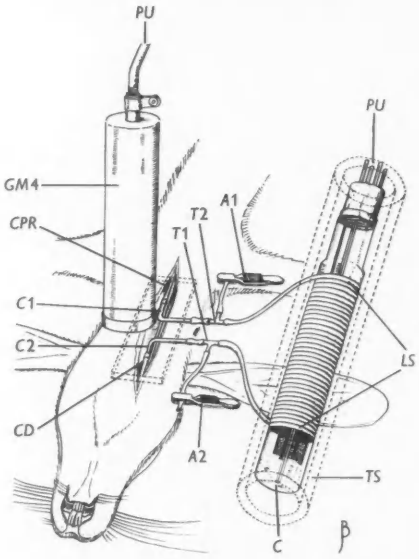


Fig. 4. Schematic diagram of the arrangement for simultaneous recording of tracers from blood stream and thyroid. *PU*, leads to probe unit; *GM* 4, G.E.C. Geiger-Müller counter inside lead shield; *C*, 20th Century Electronics counter, tungsten helix; *TS*, Telcothene spiral; *LS*, lead shield; *CPR*, proximal part of carotid; *CD*, distal part of carotid; *C* 1 and *C* 2, glass cannulae; *T* 1 and *T* 2, glass T-pieces; *A* 1 and *A* 2, artery clamps.

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